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(54) Title: METHOD OF SOLUBILIZING, PURIFYING, AND REFOLDING PROTEIN			
(57) Abstract			
<p>A method of modifying protein solubility employs polyionic polymers. These facilitate the solubilization, formulation, purification and refolding of proteins especially incorrectly folded proteins and aggregated proteins. Compositions are described that are suitable for formulating TFPI. The compositions allow preparation of pharmaceutically acceptable compositions of TFPI at concentrations above 0.2 mg/mL and above 10 mg/mL.</p>			

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METHOD OF SOLUBILIZING, PURIFYING, AND REFOLDING PROTEIN

Technical Field of the Invention

The invention relates to methods useful for refolding, solubilizing, formulating and purifying proteins. These methods are particularly useful for proteins that have been engineered by genetic recombination and produced in bacterial, yeast or other cells in a form that has a non-native tertiary structure.

Background of the Invention

To understand fully the entire process of gene expression, it is as important to understand the process for the folding of the peptide chain into a biologically active protein as it is important to understand the synthesis of the primary sequence. The biological activities of proteins depend not only on their amino acid sequences but also on the discrete conformations of the proteins concerned, and slight disturbances to the conformational integrity of a protein can destroy its activity. Tsou *et al.* (1988) *Biochemistry* 27:1809-1812.

Under the proper conditions, the *in vitro* refolding of purified, denatured proteins to achieve the native secondary and tertiary structure is a spontaneous process. To avoid formation of stable, but undesired, structures, it is necessary to use the tertiary interactions (which are formed late in folding) with their high degree of selectivity power to select and further stabilize those early local structures that are on the correct folding pathway. Thus, the finite, but very low, stability of local structures could be the kinetic "proofreading" mechanism of protein folding. The activated state of folding with the highest energy is a distorted form of the native protein, and the slowest, rate-limiting step of unfolding and refolding appears to be close to the native state in terms of ordered structure. In addition, the refolding of many proteins is not completely reversible *in vitro*, and reactivation yields of less than 100% are frequently observed, which holds true in particular for experiments at high protein concentration, and competing aggregation of unfolded or partially refolded protein molecules may be the major reason for a

- 2 -

lowered reversibility, as described in Fischer and Schmid, (1990) *Biochemistry* 29:2205-2212.

In the case of sufficiently large protein molecules, the nascent polypeptide chain acquires its native three-dimensional structure by the modular assembly of micro-domains. Variables including temperature, and cosolvents such as polyols, urea, and guanidinium chloride, have been tested to determine their role in stabilizing and destabilizing protein conformations. The action of cosolvents may be the result of direct binding or the alterations of the physical properties of water, as described in Jaenicke *et al.* (1991) *Biochemistry* 30 (13):3147-3161.

10 Experimental observations of how unfolded proteins refold to their native three-dimensional structures contrast with many popular theories of protein folding mechanisms. Under conditions which allow for refolding, unfolded protein molecules rapidly equilibrate between different conformations prior to complete refolding. The rapid prefolding equilibrium favors certain compact conformations that have somewhat 15 lower free energies than the other unfolded conformations. The rate-limiting step occurs late in the pathway and involves a high-energy, distorted form of the native conformation. There appears to be a single transition through which essentially all molecules fold, as described in Creighton *et al.* (1988) *Proc. Nat. Acad. Sci. USA* 85:5082-5086.

20 Various methods of refolding of purified, recombinantly produced proteins have been used. For example, the protease encoded by the human immunodeficiency virus type I (HIV-I) can be produced in *Escherichia coli*, yielding inclusion bodies harboring the recombinant HIV-I protease as described by Hui *et al.* (1993) *J. Prot. Chem.* 12: 323-327. The purified HIV-I protease was refolded into an active enzyme by diluting a 25 solution of the protein in 50% acetic acid with 25 volumes of buffer at pH 5.5. It was found that a higher specific activity of protease was obtained if the purified protein was dissolved at approximately 2 mg/ml in 50% acetic acid followed by dilution with 25 volumes of cold 0.1 M sodium acetate, pH 5.5, containing 5% ethylene glycol and 10% glycerol. Exclusion of glycerol and ethylene glycol led to gradual loss of protein due to precipitation. About 85 mg of correctly folded HIV-I protease per liter of *E. coli* cell culture was obtained by this method, and the enzyme had a high specific activity.

Another example of refolding a recombinant protein is the isolation and refolding of H-ras from inclusion bodies of *E. coli* as described by DeLoskey *et al.* (1994) *Arch. Biochem. and Biophys.* 311:72-78. In this study, protein concentration, temperature, and the presence of 10% glycerol were varied during refolding. The yield of correctly folded H-ras was highest when the protein was refolded at concentrations less than or equal 0.1 mg/ml and was independent of the presence of 10% glycerol. The yield was slightly higher at 4° than at 25°C.

The refolding of Tissue Factor Pathway Inhibitor (also known variously as Lipoprotein-Associated Coagulation Inhibitor (LACI), Extrinsic Pathway Inhibitor (EPI) and Tissue Factor Inhibitor (EFI) and hereinafter referred to as "TFPI") produced in a bacterial expression system has been described by Gustafson *et al.* (1994) *Protein Expression and Purification* 5: 233-241. In this study, high level expression of TFPI in recombinant *E. coli* resulted in the accumulation of TFPI in inclusion bodies. Active protein was produced by solubilization of the inclusion bodies in 8M urea, and purification of the full-length molecule was achieved by cation exchange chromatography and renaturation in 6M urea. The refolded mixture was then fractionated to yield a purified nonglycosylated TFPI possessing *in vitro* biological activity as measured in the Prothrombin clotting time assay comparable to TFPI purified from mammalian cells.

A non-glycosylated form of TFPI has also been produced and isolated from *Escherichia coli* (*E. coli*) cells as disclosed in U.S. Patent No. 5,212,091, the disclosure of which is herein incorporated by reference. The invention described in U.S. Patent No. 5,212,091 subjected the inclusion bodies containing TFPI to sulfitolysis to form TFPI-S-sulfonate, purified TFPI-S-sulfonate by anion exchange chromatography, refolded TFPI-S-sulfonate by disulfide exchange using cysteine and purified active TFPI by cation exchange chromatography. The form of TFPI described in U.S. Patent No. 5,212,091 has been shown to be active in the inhibition of bovine factor Xa and in the inhibition of human tissue factor-induced coagulation in plasma. In some assays, the *E. coli*-produced TFPI has been shown to be more active than native TFPI derived from SK hepatoma cells. However, TFPI produced in *E. coli* cells is modified in ways that increase heterogeneity of the protein.

A need exists in the art of refolding recombinantly produced proteins to increase the amount of correctly folded TFPI during the refolding process. A need also exists for increasing the solubility of TFPI. Presently the yields of recombinantly produced TFPI have been lower than desirable, and a need exists in the art of producing correctly folded 5 TFPI. See for example Gustafuson *et al.* (1994) *Protein Expression and Purification 5:* 233-241.

TFPI inhibits the coagulation cascade in at least two ways: preventing formation of factor VIIa/tissue factor complex and by binding to the active site of factor Xa. The primary sequence of TFPI, deduced from cDNA sequence, indicates that the protein 10 contains three Kunitz-type enzyme inhibitor domains. The first of these domains is required for the inhibition of the factor VIIa/tissue factor complex. The second Kunitz-type domain is needed for the inhibition of factor Xa. The function of the third Kunitz-type domain is unknown. TFPI has no known enzymatic activity and is thought to inhibit its protease targets in a stoichiometric manner; namely, binding of one TFPI 15 Kunitz-type domain to the active site of one protease molecule. The carboxy-terminal end of TFPI is believed to have a role in cell surface localization via heparin binding and by interaction with phospholipid. TFPI is also known as Lipoprotein Associated Coagulation Inhibitor (LACI), Tissue Factor Inhibitor (TFI), and Extrinsic Pathway Inhibitor (EPI).

20 Mature TFPI is 276 amino acids in length with a negatively charged amino terminal end and a positively charged carboxy-terminal end. TFPI contains 18 cysteine residues and forms 9 disulphide bridges when correctly folded. The primary sequence also contains three Asn-X-Ser/Thr N-linked glycosylation consensus sites, the asparagine residues located at positions 145, 195 and 256. The carbohydrate 25 component of mature TFPI is approximately 30% of the mass of the protein. However, data from proteolytic mapping and mass spectral data imply that the carbohydrate moieties are heterogeneous. TFPI is also found to be phosphorylated at the serine residue in position 2 of the protein to varying degrees. The phosphorylation does not appear to affect TFPI function.

30 TFPI has been isolated from human plasma and from human tissue culture cells including HepG2, Chang liver and SK hepatoma cells. Recombinant TFPI has been

expressed in mouse C127 cells, baby hamster kidney cells, Chinese hamster ovary cells and human SK hepatoma cells. Recombinant TFPI from the mouse C127 cells has been shown in animal models to inhibit tissue-factor induced coagulation.

5 A non-glycosylated form of recombinant TFPI has been produced and isolated from *Escherichia coli* (*E. coli*) cells as disclosed in U.S. Pat. No. 5,212,091. This form of TFPI has been shown to be active in the inhibition of bovine factor Xa and in the inhibition of human tissue factor-induced coagulation in plasma. Methods have also been disclosed for purification of TFPI from yeast cell culture medium, such as in Petersen *et al*, J.Biol.Chem. 18:13344-13351 (1993).

10 Recently, another protein with a high degree of structural identity to TFPI has been identified. Sprecher *et al*, Proc. Nat. Acad. Sci., USA 91:3353-3357 (1994). The predicted secondary structure of this protein, called TFPI-2, is virtually identical to TFPI with 3 Kunitz-type domains, 9 cysteine-cysteine linkages, an acidic amino terminus and a basic carboxy-terminal tail. The three Kunitz-type domains of TFPI-2 exhibit 43%,
15 35% and 53% primary sequence identity with TFPI Kunitz-type domains 1, 2, and 3, respectively. Recombinant TFPI-2 strongly inhibits the amidolytic activity of factor VIIa/tissue factor. By contrast, TFPI-2 is a weak inhibitor of factor Xa amidolytic activity.

20 TFPI has been shown to prevent mortality in a lethal *Escherichia coli* (*E. coli*) septic shock baboon model. Creasey *et al*, J. Clin. Invest. 91:2850-2860 (1993). Administration of TFPI at 6 mg/kg body weight shortly after infusion of a lethal dose of *E. coli* resulted in survival in all five TFPI-treated animals with significant improvement in quality of life compared with a mean survival time for the five control animals of 39.9 hours. The administration of TFPI also resulted in significant attenuation of the
25 coagulation response, of various measures of cell injury and significant reduction in pathology normally observed in *E. coli* sepsis target organs, including kidneys, adrenal glands, and lungs.

30 Due to its clot-inhibiting properties, TFPI may also be used to prevent thrombosis during microvascular surgery. For example, U.S. 5,276,015 discloses the use of TFPI in a method for reducing thrombogenicity of microvascular anastomoses

wherein TFPI is administered at the site of the microvascular anastomoses contemporaneously with microvascular reconstruction.

TFPI is a hydrophobic protein and as such, has very limited solubility in aqueous solutions. This limited solubility has made the preparation of pharmaceutically acceptable formulations of TFPI difficult to manufacture, especially for clinical indications which may benefit from administration of high doses of TFPI. Thus, a need exists in the art for pharmaceutically acceptable compositions containing concentrations of TFPI which can be administered to patients in acceptable amounts.

10 **Brief Description of the Drawings**

Figure 1 is a coomassie stained SDS-PAGE analysis of TFPI peak fractions from the Phenyl Sepharose HIC refolding procedure.

Figure 2 is a plot of the recovery of native TFPI from the HIC column.

Figure 3 is a plot of the recovery of native TFPI from a second HIC column.

15 Figure 4 is the amino acid sequence of TFPI.

Figure 5 shows the solubility of TFPI at different pH conditions. About 10 mg/mL TFPI in 2M urea was dialyzed against 20 mM acetate, phosphate, citrate, glycine, L-glutamate and succinate in 150 mM NaCl. The concentration of remaining soluble TFPI after dialysis was measured by UV absorbance after filtering out the precipitates through 0.22 mm filter units.

20 Figure 6 shows the solubility of TFPI as a function of concentration of citrate in the presence of 10 mM Na phosphate at pH 7. TFPI solubility increases with increasing concentration of citrate.

Figure 7 shows the solubility of TFPI as a function of concentration of NaCl. 25 TFPI solubility increases with increasing salt concentration, indicating salt promotes solubility of TFPI.

Figure 8 shows effect of pH on the stability of TFPI prepared in 10 mM Na phosphate, 150 mM NaCl and 0.005 % (w/v) polysorbate-80. Stability samples containing 150 mg/mL TFPI were incubated at 40°C for 20 days. Kinetic rate 30 constant for the remaining soluble TFPI was analyzed by following decrease of the main peak on cation exchange chromatograms.

- 7 -

Figure 9 shows the percentage of remaining soluble TFPI measured by cation exchange HPLC (A) and remaining active TFPI by prothrombin time assay (B) as a function of phosphate concentration. The formulation contains 150 mg/mL TFPI prepared in 150 mM NaCl and 0.005% (w/v) polysorbate-80 at pH 7 with varying 5 concentrations of phosphate.

Figure 10 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (triangle) and prothrombin time assay (circle) for 0.5 mg/mL TFPI formulated in 10 mM Na citrate, pH 6 and 150 mM NaCl.

Figure 11 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na phosphate, pH 6 and either 150 mM NaCl 10 (triangle) or 500 mM NaCl (circle).

Figure 12 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na acetate and pH 5.5 containing 150 mM NaCl 15 (triangle) or 8% (w/v) sucrose (square) or 4.5% mannitol (circle).

Figure 13 shows two non-reducing SDS gels for TFPI formulation samples at pH 4 to 9 stored at 40°C for 0 and 20 days.

Figure 14 shows the time course of a polyphosphate-facilitated rhTFPI refold 20 monitored using SDS PAGE.

Figure 15 shows the absorbance at 280 nm during the loading and elution of the S-Sepharose HP column used to purify rhTFPI from a polyphosphate-facilitated refold.

Figure 16 shows SDS PAGE analysis of fractions collected during elution of 25 the S-Sepharose HP column used to purify rhTFPI from a polyphosphate-facilitated refold. Figure 17 shows the absorbance at 280 nm during the loading and elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyphosphate-facilitated refold.

- 8 -

Figure 18 shows SDS PAGE analysis of fractions collected during elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyphosphate-facilitated refold.

5 Figure 19 shows the time course of a polyethyleneimine-facilitated rhTFPI refold monitored using SDS PAGE.

Figure 20 shows the absorbance at 280 nm during the loading and elution of the S-Sepharose HP column used to purify rhTFPI from a polyethyleneimine-facilitated refold.

10 Figure 21 shows SDS PAGE analysis of fractions collected during elution of the S-Sepharose HP column used to purify rhTFPI from a polyethyleneimine facilitated refold.

Figure 22 shows the absorbance at 280 nm during the loading and elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyethyleneimine-facilitated refold.

15 Figure 23 shows SDS PAGE analysis of fractions collected during elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyethyleneimine-facilitated refold.

Figure 24 shows the cation exchange HPLC analysis of a 0.4 % polyphosphate-facilitated rhTFPI refold in the absence of urea.

20 Figure 25 shows results of cation exchange HPLC analysis of an evaluation of different levels of cysteine on a rhTFPI refold in 0.4 % polyphosphate, 50 mM Tris in the absence of urea.

25 Figure 26 shows the effect of polyphosphate chain length on the course of a polyphosphate facilitated refold of rhTFPI inclusion bodies as monitored by cation exchange HPLC.

Figure 27 shows the effect of concentration of polyphosphate (Glass H) on the refolding of rhTFPI from inclusion bodies as monitored by cation exchange HPLC.

Figure 28 shows the cation exchange HPLC analysis of polyethyleneimine and polyphosphate-facilitated refolding of purified and reduced rhTFPI.

Summary of the Invention

It is an object of the present invention to describe a method of refolding protein.

It is another object of the invention to provide aqueous formulations of TFPI.

5 It is another object of the invention to provide methods for modifying a protein's solubility using charged polymers.

It is still another object of the present invention to describe a method of refolding TFPI including the steps of adding charged polymers to a solution of denatured TFPI prior to allowing the TFPI to refold.

10 Additionally, it is another object of the invention to describe a method of refolding TFPI including the step of immobilizing charged polymers on a column and passing a solution of denatured TFPI through the column and eluting the refolded TFPI after the refolding has occurred.

15 It has now been found that solubility of TFPI is strongly dependent on pH and, surprisingly, that polyanions such as citrate, isocitrate, and sulfate have profound solubilizing effects on TFPI. This finding is surprising in light of the hydrophobic nature of TFPI and the hydrophilic character of these counterions. Thus, citrate, isocitrate, sulfate as well as other solubilizers described hereinbelow can be used to produce pharmaceutically acceptable compositions having TFPI 20 concentrations sufficient for administration to patients. It has also been shown that other organic molecules can act as secondary solubilizers. These secondary solubilizers include PEG, sucrose, mannitol, and sorbitol.

The invention relates to pharmaceutically acceptable compositions wherein TFPI is present in a concentration of more than 0.2 mg/mL solubilizing agents. The 25 solubilizing agents may be acetate ion, sodium chloride, citrate ion, isocitrate ion, glycine, glutamate, succinate ion, histidine, imidazole and sodium dodecyl sulfate (SDS) as well as charged polymers. In some compositions, TFPI may be present in concentrations of more than 1 mg/mL and more than 10 mg/mL. The composition may also have one or more secondary solubilizers. The secondary solubilizer or 30 solubilizers may be polyethylene glycol (PEG), sucrose, mannitol, or sorbitol.

- 10 -

Finally, the composition may also contain sodium phosphate at a concentration greater than 20 mM.

Although the solubility of TFPI is quite low between pH 5 and 10, it has been found that L-arginine can increase the solubility by a factor of 100. The solubility is 5 very dependent on the concentration of arginine, as 300 mM is about 30 times more effective than 200 mM. Urea also is quite effective in solubilization of TFPI.

Further, it has been found that aggregation of TFPI appears to be the major degradation route at neutral and basic pH conditions and that fragmentation occurs at acidic pH conditions.

10 It has also been found that active TFPI monomers can be separated away from TFPI oligomers that are produced during the process of folding recombinant TFPI produced *E. coli*. Some misfolded/modified monomeric forms of TFPI are also removed during this process. The separation employs hydrophobic interaction chromatography. The oligomeric species of TFPI bind more tightly to a hydrophobic 15 resin than does the active TFPI monomers. Resins such as Pharmacia™ octyl sepharose and Toyopearl™ butyl 650-M have been successful. The process is carried out in the presence of high salt, such as 1 M ammonium sulfate or 0.5 M sodium citrate.

Detailed Description of the Invention

20 It is a discovery of the present inventors that polyionic polymers such as polyethylene imine and polyphosphate, can modify the ionic interactions within proteins. The masking of certain areas of high charge density within proteins using polyions can have numerous effects. Proteins whose solubility is reduced through the intra- and/or inter- molecular neutralization of oppositely charged areas can have their 25 solubility improved by masking one of the charged regions with polycations or polyanions. Barriers to conformational flexibility and specific attractive or repulsive forces which interfere with the refolding process can be modulated as well. Proteins which require strong denaturants such as urea or guanidine hydrochloride to solubilize and maintain solubility during purification operations can be solubilized and 30 processed effectively using polyions.

Many proteins lacking a clear region of charge localization in the primary sequence can still demonstrate areas of charge localization due to their secondary structure. Thus many proteins can have their solubility, refolding, and purification characteristics modified through interaction with charged polymeric templates. The 5 nature of the modification will depend on the specific protein structure, the chain length, charge, and charge density of the ionic polymer.

We have characterized refolding of pure TFPI in a guanidine or a urea based refolding buffer and the results indicate that refolding efficiencies and kinetics can be significantly improved by the addition of charged polymers including heparin, dextran 10 sulfate, polyethyleneimine (PEI) and polyphosphates. These polymers increase TFPI solubility and enhance refolding through ionic interactions with either the N-terminus or the C-terminus. In addition to the polymer additives, refolding pure TFPI requires a cysteine/cystine redox buffer where the refolding reaction can be completed within 15 48 hours. Refolding yields are a strong function of pH, redox concentration, and polymer additives; however refolding efficiencies as high as 60% can be achieved for pure TFPI under optimum refolding conditions.

It has been found by the inventors of this invention that addition of glycosaminoglycans or sulfated polysaccharides such as, for example, heparin and dextran sulfate, to a solution containing a denatured protein prior to refolding 20 increases the amount of correctly folded, active protein where the protein is capable of binding to the glycosaminoglycan or sulfated polysaccharide and is subjected to renaturing conditions.

Dissolution

25 Recombinant DNA technology has allowed the high level expression of many proteins that could not normally be isolated from natural sources in any appreciable quantities. In *E. coli* and several other expression systems, the protein is frequently expressed in an inactive, denatured state where the primary amino acid sequence is correct, but the secondary and tertiary structure and any cysteine disulfide bonds are 30 not present. The denatured protein present in an inclusion body may be in such a

- 12 -

conformation that charged residues of different parts of the amino acid backbone that are not normally in contact are able to interact and form strong ionic bonds between positively charged and negatively charged amino acid residues. The formation of these ionic bonds may limit the hydration that must occur to effect dissolution of the inclusion body. The protein in an inclusion body may also be complexed with other cellular components such as membrane components and nucleic acid which may also limit the access of solvent (water) to charged and normally hydrated residues. Also found in the unfolded state is that hydrophobic residues which are normally found buried in the interior of a protein are more exposed to the polar aqueous environment.

Such occurrences may work to prevent the dissolution of inclusion bodies in solvents other than strong chaotropic agents such as urea or guanidine or detergents such as SDS.

Charged polymers preferably in aqueous solution can interfere with and disrupt the undesirable ionic interactions that occur within a polypeptide chain as found in an inclusion body or other environment. The charged polymers may help disrupt the undesirable ionic interactions and facilitate solvation of ionic and polar residues, promoting dissolution without the need for strong chaotropes or detergents. The charge, charge density, and molecular weight (chain length) of the charged polymer may vary depending on the specific protein. Suitable polymers include: sulfated polysaccharides, heparins, dextran sulfates, agarpectins, carboxylic acid polysaccharides, alginic acids, carboxymethyl celluloses, polyinorganics, polyphosphates, polyaminoacids, polyaspartates, polyglutamates, polyhistidines, polyorganics, polysaccharides, DEAE Dextran, polyorganic amines, polyethyleneimines, polyethyleneimine celluloses, polyamines, polyamino acids, polylysines, and polyarginines.

Proteins with pI greater than 7 may benefit more from interactions with negatively charged polymers, as these proteins will have a positive charge at pH 7. Proteins with pIs below 7 may interact more strongly with positively charged polymers at neutral pH. Changing the solution pH will modify the total charge and charge distribution of any protein, and is another variable to be evaluated.

Refolding

Recombinant DNA technology has allowed the high level expression of many proteins that could not normally be isolated from natural sources in any appreciable quantities. In *E. coli* and several other expression systems, the protein is frequently expressed in an inactive, denatured state where the primary amino acid sequence is correct, but the secondary and tertiary structure and any cysteine disulfide bonds are not present. The denatured protein must be refolded to the proper active conformation, which often requires overcoming significant energy barriers imposed by ionic attraction and repulsion, restrictions on bond rotation, and other types of conformationally induced stresses. Specific ionic attraction between opposite charges and/or repulsion between like charges can severely limit the refolding pathways available to the denatured protein and reduce the efficiency of the refolding process.

Some proteins have specific areas of charge whose interactions may limit conformational flexibility or promote aggregation. Many proteins lacking a clear region of charge localization in the primary sequence can still demonstrate areas of charge localization due to their secondary structure found in refolding intermediates, misfolds and improperly folded protein. Proteins which are particularly suitable according to the present invention include but are not limited to TFPI, TFPI muteins, TFPI-2, tissue plasminogen activator, BST, PST. While it is believed that these methods will be suitable for use with proteins generically, those which are most suitable are those which are improperly folded, aggregated, oligomerized, or inactive. These will likely be proteins which have at least one highly charged domain, and possibly more, which can interact. In the case of TFPI, as well as other proteins, two oppositely charged domains interact with each other to prohibit proper folding and to cause oligomerization and aggregation. Proteins having many disulfide bonds will also be most likely to benefit from the present methods. Preferably the protein will have at least 2 and more preferably the protein will have at least 4 or 6 disulfide bonds.

Charged polymers can be used to modify the charge, charge density, and reduce or eliminate ionically mediated limitations to conformation that may arise in the unfolded state. The juxtaposition of charged groups that are not normally in proximity may have result in dead-end refolding pathways from which the refolding process may never recover.

The introduction of extra positive or negative charges through the complexation with charged polymers may allow refolding to proceed more facilely for several reasons: first different types of charge distributions may better accommodate the refolding process; second, the addition of the charged polymer may enhance the solubility of the unfolded protein, reducing or eliminating the need for chaotropes which have a negative effect on protein conformation.

Because of the frequently unique structure associated with most proteins the charged polymer that demonstrates preferred characteristics may vary. Evaluation of the isoelectric pH (pI) of the protein can serve as a starting point. At neutral pH a protein with a pI less than 7 will possess a net negative charge, and will thus be more likely to bind a positively charged polymer. The protein with a pI greater than 7 will possess a net positive charge at neutral pH, and will have a stronger tendency to bind a negatively charged polymer. However, it is well established that charges are unevenly distributed around a protein, and significant charge localization can occur. The possibility of localized concentrations of charges reduce the ability to predict which type of charged polymer may be most effective for any application. Theoretically, for any protein with a specific distribution of interacting charges and conformational requirements, there exists a charged polymer of appropriate compositions in terms of molecular weight, charge, and charge distribution which would maximize refolding efficiency. Other variables, such as pH and solvent ionic strength, would also be evaluated. Initial screening would involve polyethyleneimine, DEAE dextran, dextran sulfate, and polyphosphate at several different concentrations and molecular weights. Work with rhTFPI has demonstrated the significant impact that polyphosphate chain length and concentration can have on the course of the TFPI refolding reaction. Relatively short chain length ($n=5$) produces high levels of

aggregate. The optimal polyphosphate chain length for refolding rhTFPI was approximately 25 repeating units. Longer chain length polyphosphates ($n = 75$) also produced more aggregate and less properly folded monomer.

5 **Formulation**

Proteins consist of chains of amino acids, the exact composition and sequence of which constitutes one of the primary structure determinants of the protein. The secondary determinant of protein structure is the result of the conformational guidance that the individual amino acid bonds have on protein conformation. Thirdly, 10 the specific amino acid sequence directs the formation of tertiary structures such as β -sheets and α -helices. The three dimensional nature of protein conformation often brings into proximity amino acid residues that are not normally close to each other based on the direct sequence of the polypeptide chain. The functional form of a protein is generally a modestly stable conformation held together by a combination of 15 cysteine disulfide bonds, ionic bonds, and hydrophobic and Van der Waals interactions.

In general, protein solubility can be related to the number of charged and to a lesser extent polar amino acids that make up the protein. These charged and polar groups become solvated with water molecules in the aqueous solution and this 20 interaction keeps the polypeptide chain in solution. Polypeptides with insufficient numbers of positively or negatively charged amino acids can have limited aqueous solubility. In some cases, the positive and negatively charged groups present in a protein can interact with each other, displacing the water of solvation and leading to reduced aqueous solubility. Many proteins lacking a clear region of charge 25 localization in the primary sequence can still demonstrate areas of charge localization due to their secondary structure. Thus many proteins can have their solubility modified through interaction with charged polymeric templates. The nature of the modification will depend on the specific protein structure, the chain length, charge, and charge density of the ionic polymer.

Complexation with charged polymers with relatively high charge density represents one approach to increasing the charge density of any protein. A protein with a small number of positively charged residues (lysine or arginine) can be complexed with a negatively charged polymer such as polyphosphate. Some of the 5 negatively charged groups of the polymer will interact with the positively charged groups present in the protein. The remaining charged groups on the polymer will be free to interact with the solvent, in most cases water, and effectively increase the charge density and solvation of the protein. Alternatively, a positively charged polymer such as polyethyleneimine can be used to complex with the negatively 10 charged residues of the protein. In some cases both types of charged polymers may work equally effectively, in other cases, one charge type may be more effective than others. The effectiveness of any particular charged polymer, will depend on protein amino acid composition, protein amino acid distribution, protein conformation, charged polymer charge density, charged polymer chain length, solution pH, and 15 other variables. However, it is likely that for any given protein, a complementary charged polymer that will bind to the protein and essentially increase the charge density of protein can be found that will improve the solubility characteristics of that protein in aqueous medium.

20 Definitions

The term "processing" as used herein refers to the steps involved in the purification and preparation of pharmaceutically-acceptable amounts of proteins. Processing may include one or more steps such solubilization, refolding, chromatographic separation, precipitation, and formulation.

25 The term "charged polymer" and "charged polymeric template" refer to any compound composed of a backbone of repeating structural units linked in linear or non linear fashion, some of which repeating units contain positively or negatively charged chemical groups. The repeating structural units may be polysaccharide, hydrocarbon, organic, or inorganic in nature. The repeating units may range from n 30 = 2 to n=several million

The term "positively charged polymer" as used herein refers to polymers containing chemical groups which carry, can carry, or can be modified to carry a positive charge such as ammonium, alkyl ammonium, dialkylammonium, trialkyl ammonium, and quaternary ammonium.

5 The term "negatively charged polymer" as used herein refers to polymers containing chemical groups which carry, can carry, or can be modified to carry a negative charge such as derivatives of phosphoric and other phosphorous containing acids, sulfuric and other sulfur containing acids, nitrate and other nitrogen containing acids, formic and other carboxylic acids

10 The term "polyethyleneimine" as used herein refers to polymers consisting of repeating units of ethylene imine ($\text{H}_3\text{N}^+-(\text{CH}_2-\text{CH}_2-\text{NH}_2^+)_x-\text{CH}_2-\text{CH}_2-\text{NH}_3^+$). The molecular weight can vary from 5,000 to greater than 50,000.

15 The term "polyphosphate" as used herein refers to polymers consisting of repeating units of orthophosphate linked in a phospho anhydride linkage. The number of repeating units can range from 2 (pyrophosphate) to several thousand. Polyphosphate is frequently referred to as sodium hexametaphosphate (SHMP) Other common names include Grahams salt, Calgon, phosphate glass, sodium tetrametaphosphate, and Glass H.

20 The term "refold" as used herein refers to the renaturation of protein. Typically, the goal of refolding is to produce a protein having a higher level of activity than the protein would have if produced without a refolding step. A folded protein molecule is most stable in the conformation that has the least free energy. Most water soluble proteins fold so that most of the hydrophobic amino acids are in the interior part of the molecule, away from the water. The weak bonds that hold a protein together can be disrupted by a number of treatments that cause a polypeptide to unfold, i.e. denature. A folded protein is the product of several types of interactions between the amino acids themselves and their environment, including ionic bonds, Van der Waals interactions, hydrogen bonds, disulfide bonds and covalent bonds.

The term "denature" as used herein refers to the treatment of a protein or polypeptide in which results in the disruption of the ionic and covalent bonds and the Van der Waals interactions which exist in the molecule in its native or renatured state. Denaturation of a protein can be accomplished, for example, by treatment with

5 8 M urea, reducing agents such as mercaptoethanol, heat, pH, temperature and other chemicals. Reagents such as 8 M urea disrupt both the hydrogen and hydrophobic bonds, and if mercaptoethanol is also added, the disulfide bridges (S-S) which are formed between cysteines are reduced to two -S-H groups. Refolding of proteins which contain disulfide linkages in their native or refolded state may also involve the

10 oxidation of the -S-H groups present on cysteine residues for the protein to reform the disulfide bonds.

The term "glycosaminoglycan" as used herein refers to polysaccharides containing alternating residues of uronic acid and hexosamine and usually contain sulfate. The binding of a protein in a refolding reaction as described herein to a

15 glycosaminoglycan is through ionic interactions.

The term "dextran sulfate" as used herein refers a polyanionic derivative of dextran, ranging in molecular weight from 8,000 to 500,000 daltons. Dextrans are polymers of glucose in which glucose residues are joined by α 1,6 linkages.

The term "heparin" as used herein refers to 2 glucoaminoglycans or

20 heparinoids which are based on a repeating disaccharide (-4DGlcA(p) β 1, 4GlcNAc α 1-)_n but are subject to extensive modification after assembly. Heparin is stored with histamine in mast cell granules and is thus found in most connective tissues. In general heparins have shorter chains than heparin.

The term "HIC" as used herein refers to hydrophobic interaction chromatography

25 which employs a hydrophobic interaction between the column and the molecule of interest to separate the sulfated polysaccharides and other contaminants from the refolded product.A.

Negatively charged polymers include sulfated polysaccharides, such as heparins, dextran sulfates, and agarpectins, as well as carboxylic acid polysaccharides such

30 as alginic acids and carboxymethyl celluloses. Polyinorganics such as polyphosphates are

also included. Polyamino acids such as polyasparatate, polyglutamate, and polyhistidine can also be used.

Positively charged polymers include polysaccharides such as DEAE dextran, polyorgnic amines, such as polyethyleneimines, polyethyleneimine celluloses, and polyamines, as well as the polyamino acids, polylysine and polyarginine. Combinations of polymers may be used, of either charge polarity. In addition, amphoteric co-polymers may also be used.

As used herein, "TFPI" refers to mature Tissue Factor Pathway Inhibitor. As noted above, TFPI is also known in the art as Lipoprotein Associated Coagulation Inhibitor (LACI), Extrinsic Pathway Inhibitor (EPI) and Tissue Factor Inhibitor (TFI). Muteins of TFPI which retain the biological activity of TFPI are encompassed in this definition. Further, TFPI which has been slightly modified for production in bacterial cells is encompassed in the definition as well. For example, a TFPI analog has an alanine residue at the amino-terminal end of the TFPI polypeptide has been produced in *Escherichia coli*. See U.S. 5,212,091.

As used herein, "pharmaceutically acceptable composition" refers to a composition that does not negate or reduce the biological activity of formulated TFPI, and that does not have any adverse biological effects when formulated TFPI is administered to a patient.

As used herein, "patient" encompasses human and veterinary patients.

As used herein, the term "solubilizer" refers to salts, ions, carbohydrates, amino acids and other organic molecules which, when present in solution, increase the solubility of TFPI above 0.2 mg/mL. Solubilizers may also raise the concentrations of TFPI above 1 mg/mL and above 10 mg/mL. It should be noted that solubilizers may act as stabilizing agents. Stabilizing agents preserve the unit activity of TFPI in storage and may act by preventing formation of aggregates, or by preventing degradation of the TFPI molecule (e.g. by acid catalyzed reactions).

As used herein, the term "secondary solubilizers" refers to organic salts, ions, carbohydrates, amino acids and other organic molecules which, when present in solution with a solubilizer, further increase the solubility of TFPI. Secondary solubilizers may

have other effects as well. For example, secondary stabilizers may be useful in adjusting tonicity (e.g. to isotonicity).

The amino acid sequence of TFPI is disclosed in U.S. Patent No. 5,106,833 which is herein incorporated by reference and Figure 4. Muteins of TFPI and TFPI-2 are disclosed in U.S. Serial No. 08/286,530 which is herein incorporated by reference. As described in U.S. Serial No. 08/286,530, muteins of TFPI and TFPI-2, with single or multiple point mutations, and chimeric molecules of TFPI and TFPI-2 can be prepared. For instance, the lysine residue in the P1 site of the first Kunitz-type domain of TFPI may be replaced with arginine. Muteins, containing one to five amino acid substitutions, may be prepared by appropriate mutagenesis of the sequence of the recombinant cloning vehicle encoding TFPI or TFPI-2. Techniques for mutagenesis include, without limitation, site specific mutagenesis. Site specific mutagenesis can be carried out using any number of procedures known in the art. These techniques are described by Smith (1985) *Annual Review of Genetics*, 19:423, and modifications of some of the techniques are described in METHODS IN ENZYMOLOGY, 154, part E, (eds.) Wu and Grossman (1987), chapters 17, 18, 19, and 20. A preferred procedure when using site specific mutagenesis is a modification of the Gapped Duplex site directed mutagenesis method. The general procedure is described by Kramer, *et al.*, in chapter 17 of the Methods in Enzymology, above. Another technique for generating point mutations in a nucleic acid sequence is overlapping PCR. The procedure for using overlapping PCR to generate point mutations is described by Higuchi in Chapter 22 of PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, (eds.) Innis, Gelfand, Sninsky and White (Academic Press, 1990).

Alternatively, hybrid proteins containing the first Kunitz-type domain of TFPI-2 and the second and third Kunitz-type domains of TFPI could be produced. One skilled in the art of DNA cloning in possession of the DNA encoding TFPI and TFPI-2 would be able to prepare suitable DNA molecules for production of such a chimeric protein using known cloning procedures. Alternatively, synthetic DNA molecules encoding part or all of each Kunitz-type domain and peptide sequences linking the Kunitz-type domains can be prepared. As a further alternative, the overlapping PCR technique may

also be used to prepare DNA encoding chimeric molecules containing TFPI and TFPI-2 sequences.

TFPI can be prepared in yeast expression systems as described in U.S. Serial No. 08/286,530 which is herein incorporated by reference. Methods have also been disclosed for purification of TFPI from yeast cell culture medium, such as in Petersen *et al*, *J.Biol.Chem.* 18:13344-13351 (1993). In these cases, recombinant TFPI is secreted from the yeast cell. TFPI recovered in such protocols is also frequently heterogeneous due to N-terminal modification, proteolytic degradation, and variable glycosylation. Therefore, a need exists in the art to produce mature TFPI that is authentic (i.e. having the correct N-terminal amino acid sequence), full-length and homogeneous.

TFPI can be produced in *E. coli* as described in U.S. Patent No. 5,212,091 which discloses a method of producing TFPI by expression of a non-glycosylated form of TFPI in an *E. coli* host.

In one aspect of the invention recombinantly produced proteins which have the ability to bind polymers of sulfated polysaccharides such as, for example, heparin or dextran sulfate are refolded. The invention provides a method that facilitates refolding of a denatured recombinantly produced protein product using polymers of sulfated polysaccharides which act as a templates for the refolding protein. Without being limited to any particular theory, the inventors believe that the interactions between the refolding protein and the polymeric template may minimize aggregation of the refolding intermediates and provide an environment for the protein to refold to its native conformation. The polymer acting as a template may bind a domain or region of protein to stabilize the intermediate and allow further folding to occur without aggregation. The protein aggregates, if formed, are generally less active than non-aggregated refolded protein, and generally result in a reduced overall yield of active refolded protein. The NaCl concentration of the refolding conditions is considered important and is selected to achieve the maximum efficiency of refolding by maximizing the interaction between the template polymer and the refolding protein. For example, it has been found by the inventors that approximately 0.2 M concentration of NaCl or lower promotes binding of the C-terminal and/or the third Kunitz domain of TFPI to heparin or other sulfated polysaccharide polymer. The binding of polymer to the intermediate is presumed to

facilitate the solubility of the intermediate and provide an environment for the rest of the protein to refold by reducing aggregation of the refolding intermediates.

General Methods

5 TFPI may be prepared by recombinant methods as disclosed in U.S. 5,212,091, the disclosure of which is herein incorporated by reference. Briefly, TFPI is expressed in *Escherichia coli* cells and the inclusion bodies containing TFPI are isolated from the rest of the cellular material. The inclusion bodies are subjected to sulfitolysis, purified using ion exchange chromatography, refolded by disulfide interchange reaction and the 10 refolded, active TFPI purified by cation exchange chromatography. TFPI may also be produced in yeast as disclosed in co-pending U.S.S.N. 08/286,530.

TFPI activity may be tested by the prothrombin time assay (PTT assays). Bioactivity of TFPI was measured by the prothrombin clotting time using a model RA4 Coag-A-Mate from Organon Teknika Corporation (Oklahoma City, OK). TFPI samples 15 were first diluted to 9 to 24 ug/mL with a TBSA buffer (50 mM Tris, 100 mM NaCl, 1 mg/mL BSA, pH 7.5). Then 10 uL of Varify 1 (pooled normal plasma from Organon Teknika Corp.) was mixed with 90 uL of diluted TFPI samples in a sample tray and warmed to 37°C in the instrument. Finally Simplastin Excel (Thromboplastin from Organon Teknika Corp.) was added to start the clotting. The time delay in clotting due 20 to anticoagulant activity of TFPI was measured and converted into TFPI concentration in the measured samples by comparison to a TFPI standard curve.

The amount of soluble TFPI may also be quantified by measuring the area of the main peak on a cation exchange chromatogram. HPLC analysis of TFPI samples was performed using a Waters 626 LC system (Waters Corporation, Milford, MA) equipped 25 with a Water 717 plus heater/cooler autosampler. Data acquisition was processed by a Turbochrom™ system from Perkin-Elmer.

The cation exchange (IEX) method used a Pharmacia Mono S HR 5/5 glass column. The column was equilibrated in 80% buffer A (20 mM sodium acetate trihydrate:acetonitrile solution (70:30 v/v) at pH 5.4) and 20% buffer B (20 mM sodium 30 acetate trihydrate - 1.0 M ammonium chloride:acetonitrile solution (70:30 v/v) at pH 5.4). After a sample was injected, a gradient was applied to elute the TFPI at a flow rate

of 0.7 mL/min from 20% buffer B to 85% buffer B in 21 minutes. Eluting TFPI species were detected by absorbance at 214 nm. The main peak (monomer TFPI) was found to elute at about 18 minutes. Loss of soluble TFPI was quantified by integrating remaining peak area of the main peak.

5 All reagents are U.S.P. or A.C.S. grade. Suppliers include J.T. Baker and Sigma Co. (St. Louis, MO).

The present invention will now be illustrated by reference to the following examples, which set forth certain embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in
10 any way.

EXAMPLES

Example 1 - Refolding Denatured TFPI

15 The following example describes the making of stock solutions, the HIC column preparation, the initial recovery and purification of TFPI prior to refolding, the refolding of TFPI, and the recovery of active TFPI.

The TFPI stock was prepared from refractile bodies resulting from the expression of recombinant TFPI in bacteria. The refractile bodies were solubilized at 10 mg/ml in 8 M urea, 50 mM Tris pH 8.5 containing 10 mM DTT, and this solution was
20 clarified by centrifugation at 10,000 x g for 10 minutes.

The column preparation for the initial purification of the solubilized TFPI was prepared with S-Sepharose beads mixed in 7.5 M urea, 10 mM Tris and 10 mM sodium phosphate (pH 6.5) containing 5 mM DTT and 1 mM EDTA. The solubilized TFPI at a concentration of 5 mg/ml was then run over the S-Sepharose column and eluted with a sodium chloride gradient of 0 to 1 M. The purified TFPI had an absorbency at wavelength 280 nm of 3.2 (which is equivalent to 4.1 mg/ml using an extinction coefficient of 0.78).

30 The dextran sulfate stock consisted of dextran sulfate of molecular weight 8000 daltons available from Sigma, item number D-4911, made up at 50 mg/ml (6.25 mM) in 50 mM Tris (pH 8.8) in 0.1 M sodium chloride, and stored at -20 degrees centigrade between uses.

The heparin stock, if heparin was used to conduct the refolding, was of molecular weight 6000 to 30,000 daltons, (with an average molecular weight of 18,000 daltons) prepared as a sodium salt available from Sigma Co. (St. Louis, MO), item number H-3393, made up at 60 mg/ml (3.33 mM) in 50 mM Tris (pH8.8) in 0.1 sodium chloride, and stored at -20°C between uses.

To the S-Sepharose purified TFPI either dextran sulfate stock solution or heparin stock solution can be added. Dextran or heparin was added to TFPI under denaturing conditions in 6 to 8 M urea. With 4°C reagents, the denaturing solution containing TFPI was diluted to 3 M urea, 50 mM Tris (pH 8.8), 0.2 M sodium chloride, and 0.5 mg/ml TFPI, and to a final dextran sulfate concentration of 0.6 mg/ml (75 µM) or a final heparin concentration of 1.5 mg/ml (83 µM), depending on which was used to facilitate the refolding. Cystine was added to the refolding solution to a final concentration equal to the final DTT concentration. The refolding solution was incubated at 4°C with gentle agitation for from 4 to 6 days, preferably 5 days.

As an illustration of this procedure the following is a detail of a protocol for refolding a 5 ml solution of TFPI in dextran sulfate or heparin.

To 610 µl of TFPI stock either 60 µl of dextran sulfate with 65 µl of 50 mM Tris (pH 8.8) in 0.1 M NaCl, or 125 µl of heparin stock solution with 50 mM Tris (pH 8.8) or 0.1 M NaCl was added. The refolding solution was mixed and allowed to incubate 10 minutes on ice. Next, 4.2 ml of refolding buffer containing 2.5 M urea, 50 mM Tris (pH 8.8) and 165 mM sodium chloride was added to the refolding solution and mixed. Finally, 61 µl of 50 mM Cystine made up in 120 mM sodium hydroxide was added and the total solution was incubated at 4°C with gentle agitation for 4 days. The free sulphydryl content was checked with Ellman's reagent (also called DTNB). Iodoacetamide was added, to 20 mM, made up at 1 M in 100% ethanol for storage at -20°C.

The hydrophobic interaction column (HIC) was prepared from Butyl-650M Tosohas Toyopearl resin particle size 40-90, part # 014702. The butyl resin was washed in 3 M urea, 1 M ammonium sulfate, 50 mM Tris, 10 mM sodium phosphate, pH 6.5 and resuspended at a 50% slurry.

The refolding samples, stored at -20°C remained in the standard refolding buffer containing 3 M urea, 50 mM Tris, pH 8.8, 1-4 mM redox, 0.5 mg/ml TFPI, and 0.2-0.6 M NaCl depending on condition. Samples refolded with dextran or heparin had 0.2 M salt, and samples without dextran or heparin had 0.6 M NaCl.

5 The following steps were performed at room temperature to effect the further purification of the refolded TFPI. To 300 µl of refolded sample, an equal volume of 2 M ammonium sulfate, 3 M urea, 50 mM Tris, and 10 mM sodium phosphate (pH 6.5) was added. Next, 100 µl of washed Butyl-650M beads was added to the diluted refolded sample. The solution with the beads was incubated with gentle rocking or
10 mixing for 30 minutes at room temperature. The mix was then spun in an ependorf centrifuge for 5 seconds, and put in a rack and allowed to sit for one minute for the beads to settle flat in the tube. The supernatant was aspirated carefully, so as not to disturb the beads.

15 To wash the TFPI-bound beads, 1 ml of wash buffer composed of 1 M ammonium sulfate, 3 M urea, 50 mM Tris, 10 mM sodium phosphate (pH 6.5) was added to the beads to remove the remaining dextran sulfate or heparin. The washed mixture was re-spun in an ependorf centrifuge for 5 seconds, and allowed to sit for one minute for the beads to settle as before. The supernatant was removed, and the beads then washed with the wash buffer a final time, and spun and allowed to sit as before. After the final
20 wash and settling, the supernatant was removed with a flame-pulled-tip Pasteur pipette very carefully.

25 To elute the refolded TFPI, 300 µl of elution buffer composed of 3 M urea, 0.1 M ammonium sulfate, 50 mM Tris and 10 mM sodium phosphate (pH 6.5) was added to the slurry of beads and rocked for more than 10 minutes. The beads were pelleted by spinning in an ependorf centrifuge, and the supernatant containing refolded TFPI was recovered. To avoid contamination of the beads with the product, some of the supernatant was left behind.

Example 2 - HIC of Dextran Sulfate Refold

The sample of TFPI was renatured at a concentration of 0.5 mg/ml TFPI, 0.6 mg/ml Dextran sulfate, 3.0 M Urea, 200 mM NaCl and 50 mM Tris (pH 5.5). The HIC column was prepared from TosoHaas Butyl beads for HIC, 4.6 mmD/100mmL, in a 1.66 ml slurry. The flow rate was set for 1.0 ml/min. Before loading the HIC column, the sample was diluted 2:3 with 3.0 M Urea and 3.0 M NH₄SO₄, at a final pH of 5.68; 2 ml of sample was loaded. The gradient start was 33 mM MES/33 mM HEPES/33 mM sodium acetate, 1.0 M NH₄SO₄, and 3.0 M Urea, pH 6.0; the gradient end was 33 mM MES/33 mM HEPES/33 mM sodium acetate, 3.0 M Urea at pH 6.0. The gradient volume was 5.0 CV. From this column, the recovery of native TFPI was 68%. The results of this run are shown in Figure 2.

A second HIC column was also run. The sample of denatured TFPI was diluted 2:3 with 3.0 M Urea, 1.5 M NH₄SO₄, and two ml were loaded. The gradient start was 33 mM MES/33 mM HEPES/33 mM sodium acetate, 0.5 M NH₄SO₄, and 3.0 M Urea, pH 6.0; the gradient end was 33 mM MES/33 mM HEPES/33 mM sodium acetate, 3.0M Urea at pH 6.0. The gradient volume was 5.0 CV. The recovery of native TFPI from this second column was 74%. The results of this run are shown in Figure 3.

The samples were analyzed by non-reducing SDS-PAGE as illustrated in Figure 1. Correctly refolded, active TFPI species (major band) are seen on the gel.

Example 3

About 10 mg/mL TFPI in 2M urea was dialyzed against one of the following: 20 mM acetate, 20 mM phosphate, 20 mM citrate, 20 mM glycine, 20 mM L-glutamate or 20 mM succinate in 150 mM NaCl as described above. 6-10 mg/mL TFPI bulk stock was loaded into Spec/Por 7 dialysis tubings (MW cutoff 3,500). Dialysis was carried out either at 4°C or ambient temperature. Three changes of buffer at a protein solution to buffer ratio: 1 to 50-100, were made during course of dialysis over 12 to 24 hr time period. After dialysis, TFPI solution was filtered by Costar 0.22 micron filter units to separate precipitated TFPI from soluble TFPI. The solubility of TFPI was then measured by UV/Vis absorbance assuming an absorptivity 0.68 (mg/mL)⁻¹ cm⁻¹ at 278 nm. The solutions were prepared at various pH levels by titration with HCl or NaOH.

After completion of dialysis, the precipitates were filtered through 0.22 μ m filter units. The concentration of remaining soluble TFPI after dialysis was measured by UV absorbance. Figure 1 shows the results of these experiments. Solubility of TFPI increased greatly in solutions containing 20 mM acetate, 20 mM phosphate, 20 mM L-glutamate and 20 mM succinate at pH levels below 7 and particularly at or below pH 4.5. Solubility of TFPI was also substantially increased in solutions containing 20 mM glycine above pH 10. Figure 2 shows the solubility of TFPI as a function of concentration of citrate ion in the presence of 10 mM Na phosphate at pH 7. TFPI solubility increases with increasing concentration of citrate. Figure 3 shows the solubility of TFPI as a function of concentration of NaCl at pH 7.0. TFPI solubility increases with increasing salt concentration, indicating salt promotes solubility of TFPI.

The solubility of TFPI was studied using a number of different solubilizers and secondary solubilizers. Table 1 shows solubility of TFPI in varying buffer solutions measured by UV absorbance after dialyzing 6 to 10 mg/mL TFPI into these buffer solutions.

Table 1

Salt effect		Solubility	
Content	pH	c (mg/ml)	uv
10mM NaPO ₄ ,	7	0.21	
10mM NaPO ₄ , 150mM NaCl	7	0.72	
20mM NaPO ₄ , 150mM NaCl	7	0.85	
20mM NaPO ₄ , 0.5M NaCl	7	6.71	
20mM NaPO ₄ , 1M NaCl	7	8.24	
pH effect			
Content	pH	c (mg/ml)	uv
20mM NaOAc, 150mM NaCl	3	10.27	
20mM NaOAc, 150mM NaCl	3.5	10.25	
20mM NaOAc, 150mM NaCl	4	7.54	
20mM NaOAc, 150mM NaCl	4.5	1.75	
20mM NaOAc, 150mM NaCl	5	1.15	
20mM NaOAc, 150mM NaCl	5.5	0.85	
20mM NaPO ₄ , 150mM NaCl	5.5	0.89	
20mM NaPO ₄ , 150mM NaCl	6	0.78	
20mM NaPO ₄ , 150mM NaCl	6.5	0.79	
20mM NaPO ₄ , 150mM NaCl	7	0.95	
20mM NaPO ₄ , 150mM NaCl	7.5	0.82	
20mM NaPO ₄ , 150mM NaCl	8	0.86	
20mM NaCitrate, 150mM NaCl	4	2.17	
20mM NaCitrate, 150mM NaCl	4.5	1.19	
20mM NaCitrate, 150mM NaCl	5	1.1	
20mM NaCitrate, 150mM NaCl	5.5	1.84	
20mM NaCitrate, 150mM NaCl	6	2.09	
20mM NaCitrate, 150mM NaCl	6.5	2.12	
20mM NaCitrate, 150mM NaCl	7	1.92	
20mM Glycine, 150mM NaCl	9	0.32	
20mM Glycine, 150mM NaCl	10	0.9	
20mM Glycine, 150mM NaCl	11	13.94	
20mM L-Glutamate, 150mM NaCl	4	9.07	
20mM L-Glutamate, 150mM NaCl	5	1.21	
20mM Succinate, 150mM NaCl	4	8.62	
20mM Succinate, 150mM NaCl	5	1.21	
20mM Succinate, 150mM NaCl	6	1.07	
Citrate			
Content	pH	c (mg/ml)	uv
10mM NaPO ₄ , 20mM NaCitrate	7	1.16	

- 29 -

Table 1 (cont.)

10mM NaPO ₄ , 50mM NaCitrate	7	5.81
10mM NaPO ₄ , 100mM NaCitrate	7	12.7
10mM NaPO ₄ , 200mM NaCitrate	7	15.9
10mM NaPO ₄ , 300mM NaCitrate	7	8.36
Mg²⁺, Ca²⁺ and polyphosphate		
Content	pH	c (mg/ml) uv
10mM NaPO ₄ , 150mM NaCl, 1mM MgCl ₂	7	0.66
10mM NaPO ₄ , 150mM NaCl, 10mM MgCl ₂	7	1.02
10mM NaPO ₄ , 150mM NaCl, 0.1mM CaCl ₂	7	0.67
10mM NaPO ₄ , 150mM NaCl, 1mM CaCl ₂	7	0.71
10mM NaPO ₄ , 150mM NaCl, 10mM triphosphate	7	3.64
10mM NaPO₄, 5% PEG-400		
10mM NaPO ₄ , 10mM EDTA	7	0.07
10mM NaPO ₄ , 100mM Na ₂ SO ₄	7	0.36
10mM NaPO ₄ , 100mM L-aspartic acid	7	5.08
10mM NaPO ₄ , 100mM Succinic acid	7	0.4
10mM NaPO ₄ , 100mM Tartaric acid	7	2.33
20mM NaPO ₄ , 100mM Maleic acid	7	2.56
20mM NaPO ₄ , 100mM Malic acid	7	0.11
10mM NaPO ₄ , 100mM L-glutamic acid	7	1.87
10mM NaPO ₄ , 150mM NaCl	7	0
10mM NaPO ₄ , 100mM isocitrate	7	0.25
10mM NaPO₄, 100mM isocitrate		
NaOAc, NaPO₄ and NaCl		
Content	pH	c (mg/ml) uv
10mM NaOAc, 150mM NaCl	4.5	1.76
10mM NaOAc	4.5	4.89
10mM NaOAc	5.5	4.95
10mM NaOAc	6.5	5.1
10mM NaOAc	7	5.87
10mM NaPO ₄ , 150mM NaCl	4.5	0.14
10mM NaPO ₄	4.5	4.97
10mM NaPO ₄	5.5	0.79
10mM NaPO ₄	6.5	0.091
10mM NaPO ₄	7	0.94
50mM NaOAc		
50mM NaOAc	5	5.24
5mM NaOAc		
5mM NaOAc	5.5	4.59
10mM NaOAc	5.5	5.05
20mM NaOAc	5.5	5.04
50mM NaOAc	5.5	5.71
100mM NaOAc	5.5	1.4
200mM NaOAc	5.5	1.32

- 30 -

Table 1 (cont..)

5mM NaOAc, 5mM NaCl	5.5	4.85
5mM NaOAc, 10mM NaCl	5.5	5.04
5mM NaOAc, 50mM NaCl	5.5	0.56
5mM NaOAc, 100mM NaCl	5.5	0.43
5mM NaOAc, 200mM NaCl	5.5	0.8
5mM NaOAc	4.5	7.27
10mM NaOAc	4.5	6.5
20mM NaOAc	4.5	8.32
50mM NaOAc	4.5	9.17
5mM NaOAc	5.5	8.98
10mM NaOAc	5.5	8.08
20mM NaOAc	5.5	8.99
50mM NaOAc	5.5	2.92
5mM NaOAc, 150mM NaCl	4.5	2.6
10mM NaOAc, 150mM NaCl	4.5	2.59
20mM NaOAc, 150mM NaCl	4.5	2.55
50mM NaOAc, 150mM NaCl	4.5	2.1
5mM NaOAc, 150mM NaCl	5.5	0.65
10mM NaOAc, 150mM NaCl	5.5	0.69
20mM NaOAc, 150mM NaCl	5.5	0.74
50mM NaOAc, 150mM NaCl	5.5	0.91
Hydrophobic chain length		
Content	pH	c (mg/ml) uv
10mM NaPO4, 50mM Formic acid	7	0.12
10mM NaPO4, 50mM Acetic acid	7	0.16
10mM NaPO4, 50mM Propanoic acid	7	0.16
10mM NaPO4, 50mM Butanoic acid	7	0.13
10mM NaPO4, 50mM Pentanoic acid	7	0.14
10mM NaPO4, 50mM Hexanoic acid	7	0.11
Others		
Content	pH	c (mg/ml) uv
20mM NaOAc, 3% Mannitol, 2% Sucrose, 5% PEG-400	4	19.9
20mM Na Citrate, 3% Mannitol, 2% Sucrose, 5% PEG-400	6.5	0.72
20mM Na Citrate, 150mM NaCl, 5% PEG-400	6.5	2.18
20mM NaOAc, 150mM NaCl, 5% PEG-400	4	19.8
20mM Na Citrate, 130mM NaCl, 1% Glycine, 0.25% Tween-80, 5% PEG-400	6.5	1.48
20mM Na Citrate, 130mM NaCl, 1% Glycine, 0.25% Tween-80	6.5	1.32
		Solubility
Content	pH	c (mg/ml),uv
5mM NaAcetate	5.5	8.9
5mM NaAcetate, 8% Sucrose	5.5	11
5mM NaAcetate, 0.01% Polysorbate-80	5.5	7

Table 1 (cont.)

5mM NaAcetate, 8% Sucrose, 0.01% Polysorbate-80	5.5	12
10mM NaAcetate	5.5	7.6
10mM NaAcetate, 8% Sucrose	5.5	10
10mM NaAcetate, 8% Sucrose, 0.01% Polysorbate-80	5.5	12.1
5mM NaAcetate, 5% Sorbitol	5.5	7.8
5mM NaAcetate, 4.5% Mannitol	5.5	9.2
5mM Histidine	6	5.5
5mM Histidine	6.5	1
5mM NaCitrate	5.5	0.1
5mM NaCitrate	6	0.1
5mM NaCitrate	6.5	0.1
5mM NaSuccinate	5.5	0.6
5mM NaSuccinate	6	0.3
5mM NaSuccinate	6.5	0.2
10mM Imidazole	6.5	2.5, 10.8
10mM Imidazole	7	0.8
10mM Imidazole, 8% Sucrose	6.5	12.2
5mM NaAcetate	6	8.2
10mM Imidazole, 5mM NaAcetate	6.5	12.8
10mM NaCitrate	6	0.2
100mM NaCitrate	6	8.1
100mM NaCitrate	7	9.3
10mM Naphosphate, 260mM Na ₂ SO ₄	6	9.1
10mM NaPhosphate, 100mM NaCitrate	8	8.8
10mM NaCitrate, 1% L-glutamic acid	6	4.6
10mM NaCitrate, 2% L-lysine	6	1.1
10mM NaCitrate, 0.5% L-aspartic acid	6	0.4
10mM NaCitrate, 0.1% Phosphate glass	7	5.9
10mM Tris, 100mM NaCitrate	8	8.5
10mM NaCitrate, 1M Glycine	6	0.3
10mM NaCitrate, 300mM Glycine	6	0.3
10mM NaCitrate, 280mM Glycerol	6	0.3
10mM NaCitrate, 0.5M (NH ₄) ₂ SO ₄	6	8.3
10mM NaCitrate, 120mM (NH ₄) ₂ SO ₄	6	8.8
10mM NaCitrate, 260mM Na ₂ SO ₄	6	9.4
10mM NaPO ₄ , 0.1% Phosphate glass	7	15.8
10mM NaCitrate, 0.1% SDS	6	11.2
10mM NaCitrate, 0.02% SDS	6	7.8
10mM NaAcetate, 8% PEG-400	5.5	13.7
10mM NaAcetate, 150mM NaCl, 8% PEG-400	5.5	0.6
10mM NaAcetate, 8% PEG-400	6	16.2
10mM NaCitrate, 8% PEG-400	6	0.2

Example 4

The stability of TFPI stored at various pH conditions was tested. TFPI was prepared by dialysis as above in 10 mM Na phosphate, 150 mM NaCl and 0.005% (w/v) polysorbate-80. Stability samples containing 150 mg/mL TFPI were incubated 5 at 40°C for 20 days. Kinetic rate constant for the remaining soluble TFPI was analyzed by following decrease of the main peak on cation exchange chromatograms. As can be seen in Figure 5, the decay rate constant increases at pH above 6.0, indicates more aggregation at higher pH conditions.

TFPI was also formulated at a concentration of 150 mg/mL in 150 mM NaCl 10 and 0.005% (w/v) polysorbate-80 at pH 7 with varying concentrations of phosphate. Figure 5A shows the percentage of remaining soluble TFPI measured by the cation exchange HPLC. Increasing concentrations of phosphate ion in solution resulted in higher levels of soluble TFPI remaining after incubation at 40°C. Higher levels of phosphate ion also resulted in higher levels of active TFPI as assayed by the 15 prothrombin time assay. These results are shown in Figure 5B.

Stability of TFPI at a concentration of 0.5 mg/mL and formulated in 10 mM Na citrate, pH 6 and 150 mM NaCl was also tested at 40°C over a 40 day period. As seen in Figure 6, cation-exchange HPLC (triangle) shows the presence of soluble 20 TFPI at levels greater than 60% initial, even after the 40 day incubation. In like manner, the prothrombin time assay (circle) shows the presence of active TFPI at levels greater than 60% initial, even after the 40 day incubation.

Figure 7 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na phosphate, pH 6 and either 150 mM NaCl 25 (triangle) or 500 mM NaCl (circle).

Figure 8 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na acetate and pH 5.5 containing 150 mM NaCl (triangle) or 8% (w/v) sucrose (square) or 4.5% (w/v) mannitol (circle).

Figure 9 shows two non-reducing SDS gels for TFPI formulation samples in 10 mM NaPO₄, 150 mM NaCl, and 0.005% polysorbate-80 at pH 4 to pH 9 stored at 40°C for 0 days (lower) and 20 days (upper). No loss on TFPI is seen at 0 days. However, at 20 days cleavage fragments of TFPI may be seen at the lower pH range (i.e. pH 4 and pH 5). Without being bound to a particular theory, it is believed that these fragments may result from an acid catalyzed reaction.

Finally, Table 2 shows the half-life of remaining soluble TFPI at 40°C for various formulations. 0.5 mg/mL TFPI was formulated in these formulation conditions and incubated at 40°C. Samples were withdrawn at predetermined time intervals and loss of soluble and active TFPI were examined by the IEX-HPLC and the PT assay. Half-life for remaining soluble TFPI was then calculated by performing a single exponential fitting to the IEX-HPLC and PT assay results.

Example 5

Elution of TFPI in displacement mode from chromatography resins using polyionic compounds.

TFPI is first bound to a resin in a low salt buffer. Next a buffer containing the polyionic compound used to elute TFPI in displacement mode, is pumped through the column. This compound binds stronger to the resin than TFPI and displaces TFPI. For a positively charged resin (anion exchanger) a negatively charged compound is used and for a negatively charged resin (cation exchanger) a positively charged compound is used.

Partially purified TFPI was used as starting material. TFPI, in 6 M urea, 20 mM Tris, pH 8.0 was loaded onto a column packed with an anion exchange resin, Q Sepharose HP, to 20 mg/mL resin. After loading, the column was washed with 6 M urea, 20 mM Tris, pH 9.0. TFPI was eluted and 10 mg/ml of Glass H (polyphosphate) in 6 M urea, 10 mM Tris, pH 9.0.

Example 6

Elution of TFPI from chromatography resin in aqueous buffer using polyionic compounds.

Table 2

0.5 mg/ml TFPI formulated in:	t _{1/2} (day) at 40°C	
	IEX-HPLC	PT assay
10 mM Na Acetate, 150 mM NaCl, pH 5.5	10.8	17.2
10 mM Na Citrate, 150 mM NaCl, pH 5.5	12.2	24.4
10 mM Na Acetate, 8% (w/v) Sucrose, pH 5.5	43.2	42.2
10 mM Na Acetate, 4.5% Mannitol, pH 5.5	47.7	46.6
10 mM Na Succinate, 150 mM NaCl, pH 6.0	7.8	11.0
10 mM Na Citrate, 150 mM NaCl, pH 6.0	13.0	18.8
10 mM Na Phosphate, 150 mM NaCl, pH 6.0	7.8	11.2
10 mM Na Phosphate, 500 mM NaCl, pH 6.0	52.2	68.9
10 mM Na Citrate, 150 mM NaCl, pH 6.5	10.0	14.8

For a positively charged resin, a positively charged compound is used and for a negatively charged resin, a negatively charged compound is used.

TFPI, in 3.5. M urea, 1 mg/ml polyphosphate, 50 mM Tris, pH 5.9 was loaded onto a cation exchange resin, SP Sepharose HP. After loading the column was washed with a non-urea containing buffer, 10 mg/ml polyphosphate, 10 mM sodium phosphate, pH 5.0. TFPI was eluted in the same buffer at pH 7.5, without urea.

Example 7

Selective elution of TFPI from ion exchange resins using polyionic compounds.

Because of the charged ends of TFPI, oppositely charged polyionic compounds can bind to these ends. When the polyionic compound has a higher strength of binding to TFPI than does the resin, the TFPI may be selectively eluted from the chromatography resin.

TFPI, in 3.5. M urea, 1 mg/ml polyphosphate, 50 mM Tris, pH 5.9 was loaded onto a cation exchange resin, SP Sepharose HP. After loading, the column is washed with 6 M urea, 1mg/ml polyphosphate, 10 mM sodium phosphate, pH 5.9. TFPI was eluted in a 25 column volume gradient up to 20 mg/ml of polyphosphate. TFPI starts to elute at about 2-3 mg/ml of polyphosphate.

Example 8

Neutralization of polyionic compounds prior to chromatographic separation of TFPI. TFPI can interact with charged polymers. This interaction may prevent binding and purification to chromatographic resins. By neutralizing the charged polymer with an oppositely charged polymer, TFPI may bind to the resin.

In a buffer containing polyphosphate (Glass H), TFPI does not bind to Express Ion S (Whatman)and no purification is achieved. By mixing PEI into the column load, TFPI now binds to the resin and TFPI can be purified.

Example 9

Refolding and purification of recombinant human TFPI (rhTFPI) using Polyphosphate (Glass H) Facilitated Refolding Process.

Inclusion bodies containing about 40 g of rhTFPI were thawed by removing
5 the containers from the -20°C freezer and incubating them in a cold room at 4-10°C
for approximately 196 hours. The thawed inclusion bodies were then dispersed with
a high shear mixer to reduce the clumping that occurs during freezing. The thawed
inclusion bodies were added to 80 L of 3 M urea, 50 mM Tris-Cl, pH 10.5 buffer
containing 2 g/L Glass H contained in a 100 L polyethylene tank equipped with an
10 overhead stirrer. The contents were mixed for approximately 15 minutes, and then
the absorbance of the solution is measured at 280 nm. If the absorbance is greater
than the mixture was diluted with sufficient dissolution buffer to obtain an absorbance
at 280 nm of 1.0-1.1. The solution was incubated with gentle agitation for 15-30
minutes, and then sufficient cysteine was added to give a cysteine concentration of
15 0.1 mM. The solid L-cysteine was dissolved in approximately 50 ml of purified
water and added to the refold mixture. The pH was checked and adjusted to pH 10.2
if necessary. The refold mixture was incubated with gentle agitation for 96-120
hours.

After approximately 96 h, the refolding process was terminated by adjusting
20 the pH of the refold mixture to pH 5.9 using glacial acetic acid. Stirring was
continued for 90 minutes and the pH checked. More acid was added, if necessary to
adjust the pH to 5.9 +/- 0.1. A two-step filtration process was used to remove the
particulates that formed during previous steps and prepare the acidified refold mixture
for SP-Sepharose HP chromatography. First the acidified refold mixture is passed
25 through A Cuno 60LP depth filter (filter housing model 8ZP1P) using a peristaltic
pump ($\frac{1}{4}$ - $\frac{3}{8}$ inch inner diameter silicon tubing).

The filter system was washed with 8-10 L of deionized 6 M urea before use.
The filtrate was collected in a 100 L polyethylene tank. Back pressure was
maintained at a constant 20 PSI. Initial flow rate for a new filter was approximately
30 5-6 L per minute. Filters were replaced when the flow rate dropped below 1 L per

minute in order to maintain the back pressure at 20 PSI. The second stage of the filtration used a 0.45 micron filter cartridge (Sartorius Sartobran pH or equivalent) with a peristaltic pumping system. After filtration, the pH was checked, and adjusted to pH 5.9 if necessary.

5 The acidified, filtered refold was loaded onto the equilibrated SP Sepharose HP column at a flow rate of approximately 80.0 ml/min. Flow rate was adjusted to accommodate overnight loading of the acidified filtered refold mixture. The column was equilibrated in 6 M urea, 20 mM sodium phosphate buffer pH 5.9 prior to loading. After loading, the column is washed with 2 CF of 6 M urea, 0.3 M NaCl, 10 20 mM sodium phosphate buffer, pH 5.9 prior to the gradient elution step. The column flow rate was increased to 190-200 ml/min for the wash step and all subsequent steps (linear velocity = ~ 47 cm/hr). The product was eluted from the column using a linear salt gradient from 0.3 to 0.5 M NaCl in 6 M urea, 20 mM sodium phosphate buffer, pH 5.9. The gradient was formed by delivering 6 M urea, 15 0.5 M NaCl, 20 mM sodium phosphate buffer into 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer. Limit buffer was pumped with a Masterflex pump (model 7553-20) with a Masterflex head (model 7015.21) at a flow rate of approximately 100 ml/min. with vigorous mixing using a Paratrol A mixer from Parametrics (model 250210). The total volume of the gradient was 71.0 liters or 13.0 CV. The pH of 20 the gradient buffers was 5.92 (+/- .02). Fractions are evaluated qualitatively using SDS PAGE and pooled based on the content of the correctly folded SC-59735 relative to other misfolds and impurities. After pooling the process stream is referred to as the S pool.

25 The pH of the S pool was next adjusted to pH 8.0 with 2.5 N NaOH. The S pool was concentrated 2-3 fold to approximately 2 L using an Amicon DC-10L ultrafiltration unit containing an Amicon YM10 spiral cartridge (10,000 M.W. cut-off membrane). After concentration, the concentrated S pool was diafiltered against 7 volumes of 6 M urea, 20 mM Tris-HCl buffer, pH 8.0. The diafiltration was considered complete when the conductivity of the retentate was below 2 mS. The 30 diafiltered concentrate was drained from the ultrafiltration unit and the unit was

washed with approximately 1 L of diafiltration buffer. The was is combined with the concentrate to form the Q-load.

An Amicon column (7.0 cm diameter) was packed with approximately 700 ml of Q-Sepharose high performance medium (Pharmacia Q-Sepharose HP). The 5 column was packed with 20% ethanol at 20 psi. The bed height after packing was approximately 18 cm. The column was equilibrated with 5 CV of 6 M urea, 0.02 M Tris/HCl buffer, pH 8. The target for protein loading is 8-10 mg protein/ml Q Sepharose resin. The Q load was applied to the column at a flow rate 30-35 ml/min (50 cm/hr). After loading, the column was washed with approximately 5 CV of 6 M 10 urea, 20 mM Tris/HCl buffer, pH 8.0, or until the absorbance at 280 nm returned to baseline. The product was eluted using a sodium chloride gradient from 0-0.15 M NaCl in 6 M urea, 20 mM Tris/HCl buffer, pH 8.0 over 25 column volumes. The first seven column volumes were collected as a single fraction, followed by 30 fractions of 0.25 column volume each.

15 Fractions are routinely analyzed by reducing and non-reducing SDS-PAGE and size exclusion chromatography. Fractions are pooled based on aggregate content (<5% by SEC HPLC Method MSL 13929) and qualitative evaluation by SDS PAGE to assess purity. The fractions are stored frozen at -20°C until pooled.

Acceptable Q Sepharose fractions were pooled, and the pH of the pool was 20 adjusted to 7.2 using 2 M HCl. The pool was then concentrated approximately 5 fold in an Amicon DC-1 ultrafiltration system containing a S1Y1 Amicon YM-10 cartridge (10,000 MWCO spiral cartridge membrane). The concentrated Q Pool was then diafiltered against seven column volumes of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2. Following ultrafiltration, the solution was drained 25 from the ultrafiltration system. Approximately 100 ml of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2 was circulated through the ultrafiltration system for approximately 5 min. The rinse solution was combined with the original concentrate and the solution was filtered through a 0.45 micron vacuum filter unit (Nalgene).

Example 10**Refolding and Purification of rhTFPI using Polyethyleneimine (PEI) Facilitated Refolding Process.**

Inclusion bodies containing about 40 g of rhTFPI were thawed by removing
5 the containers from the -20°C freezer and incubating them in a cold room at 4-10°C
for approximately 96 hours. The thawed inclusion bodies were then dispersed with a
high shear mixer to reduce the clumping that occurs during freezing. The inclusion
body slurry was vigorously blended for approximately 1 minute using a polytron
homogenizer (Brinkman model PT45/80) or until the inclusion bodies were then
10 added to 40 L of 6 M urea 100 mM Tris/HCl buffer pH 9.8 containing 300 mM
NaCl and 0.4 g/L PEI contained in a 100 L polyethylene tank equipped with an
overhead stirrer. The mixture was vigorously stirred for 20-30 min. The pH was
monitored and adjusted to pH 9.8 as necessary. The absorbance of the dissolved
inclusion body mixture was measured at 280 nm, and if the absorbance was greater
15 than 2.1, the sample was diluted with 10 liters of the dissolution buffer described
above to obtain an A280 value of 2.0-2.1. Gentle agitation was continued for another
15-30 minutes. Next, the dissolved inclusion body solution was diluted with an equal
volume of 1.0 M urea, 300 mM NaCl solution. Finally, L-cysteine was added to
give a final concentration of 0.25 mM. The solid L-cysteine was dissolved in 50 ml
20 of WFI and added as a solution to the diluted refold. The pH was checked and
adjusted, if necessary. The refold continued with gentle mixing for 96-120 hours
with periodic checks of the pH, and adjustment to pH 9.8, if necessary. The progress
of the refold was monitored by Mon-S cation exchange and prothrombin time assays.

After approximately 96 h, the refolding process was terminated by adjusting
25 the pH of the refold to pH 5.9 using glacial acetic acid. Stirring was continued for
90 minutes and the pH checked. More acid was added, if necessary to adjust the pH
to 5.9 +/- 0.1.

A two-step filtration process was used to remove the particulates that formed
during previous steps and prepare the acidified refold for SP-Sepharose HP
30 chromatograph. First, the acidified refold is passed through a Cuno 60LP depth filter

- 40 -

(filter housing model 8ZP1P) using a peristaltic pump ($\frac{1}{4}$ - $\frac{3}{8}$ inch inner diameter silicon tubing).

The filter system was washed with 8-10 L of deionized 6 M urea before use. The filtrate was collected in a 100 L polyethylene tank. Back pressure was maintained at a constant 20 PSI. Initial flow rate for a new filter was approximately 5-6 L per minute. Filters were replaced when the flow rate dropped below 1 L per minute in order to maintain the back pressure at 20 PSI. The second stage of the filtration used a 0.45 micron filter cartridge (Sartorius Sartobran pH or equivalent) with a peristaltic pumping system. After filtration, the pH was checked, and adjusted to pH 5.9, if necessary.

The acidified, filtered refold was loaded onto the equilibrated SP Sepharose HP column at a flow rate of approximately 80.0 ml/min. Flow rate was adjusted to accommodate overnight loading of the acidified filtered refold. The column was then washed with 5.5 column volumes of 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer, pH 5.9. The column flow rate was increased to 190-200 ml/min for the wash step and all subsequent steps (linear velocity = ~ 47 cm/hr). The product was eluted from the column using a linear salt gradient from 0.3 to 0.5 M NaCl in 6 M urea, 20 mM sodium phosphate buffer, pH 5.9. The gradient was formed by delivering 6 M urea, 0.5 M NaCl, 20 mM sodium phosphate buffer into 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer into 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer. Limit buffer was pumped with a Masterflex pump (model 7553-20) with a Masterflex head (model 7015.21) at a flow rate of approximately 100 ml/min with vigorous mixing using a Paratrol A mixer from Parametrics (model 250210). The total volume of the gradient was 71.0 liters or 13.0 CV. The pH of the gradient buffers was 5.92 (+/- .02).

Fraction collection was started when the column inlet conductivity reached 28.0 - 28.5 mS/cm as measured by the in-line Radiometer conductivity meter. Forty 500 ml fractions (0.1 CV) were collected. A Pharmacia Frac-300 fraction collector was used with numbered, 500 ml polypropylene bottles. When the fraction collection was stopped, the remainder of the gradient was collected as a pool.

Column fractions were assayed by A280, size exclusion HPLC, and in addition, for informational purposes, SDS PAGE, reverse phase HPLC, and PT assays. Fractions were pooled if they met the pooling criteria of containing 20% or less aggregate as determined by the in process SEC HPLC. Pooled SP Sepharose 5 fractions are referred to as the S Pool.

The pH of the S-pool was next adjusted to pH 8.0 with 2.5 N NaOH. The S Pool was concentrated 2-3 fold to approximately 2 L using an Amicon DC-10L ultrafiltration unit containing an Amicon YM10 spiral cartridge (10,000 N.W. cut-off membrane). After concentration, the concentrated S Pool was diafiltered against 10 7 volumes of 6 M urea, 20 mM Tris-HCl buffer, pH 8.0. The diafiltration was considered complete when the conductivity of the retentate was below 2 mS. The diafiltered concentrate was drained from the ultrafiltration unit and the unit was washed with approximately 1 L of diafiltration buffer. The was is combined with the concentrate to form the Q-load.

An Amicon column (7.00 cm diameter) was packed with approximately 700 ml of Q-Sepharose high performance medium (Pharmacia Q-Sepharose HP). The column was packed in 20% ethanol at 20 psi. The bed height after packing was approximately 18 c.m The column was equilibrated with 5 CV of 6 M urea, 0.02 M Tris/HCl buffer, pH 8. The target for protein loading is 8-10 mg protein/ml Q 20 Sepharose resin. The Q load was applied to the column at a flow rate 30-35 ml/min (50 cm/hr). After loading, the column was washed with approximately 5 CV of 6 M urea, 20 mM Tris/HCl buffer, pH 8.0, or until the absorbance at 280 nm returned to baseline. The product was eluted using a sodium chloride gradient from 0-0.15 M NaCl in 6 M urea, 20 mM Tris/HCl buffer, pH 8.0 over 25 column volumes. The 25 first seven column volumes were collected as a single fraction, followed by 30 fractions of 0.25 column volume each.

Fractions are routinely analyzed by reducing and non-reducing SDS-PAGE and size exclusion chromatography. Fractions are pooled based on aggregate content (5% by SEC HPLC) and qualitative evaluation by SDS PAGE to assess purity. The 30 fractions are stored frozen at -20°C until pooled.

The Q-Sepharose fractions to be pooled were thawed by incubation at 2-8°C, pooled, and the pH of the pool was adjusted to 7.2 using 2 M HCl. The pool was then concentrated approximately 5 fold in an Amicon DC-1 ultrafiltration system containing a S1Y1 Amicon YM-10 cartridge (10,000 MWCO spiral cartridge membrane). The concentrated Q Pool was then diafiltered against seven column volumes of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2. Following ultrafiltration, the solution was drained from the ultrafiltration system. Approximately 100 ml of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2 was circulated through the ultrafiltration system for approximately 5 min.

5 The rinse solution was combined with the original concentrate and filtered through a 0.45 micron vacuum filter unit (Nalgene).

10 The rinse solution was combined with the original concentrate and filtered through a 0.45 micron vacuum filter unit (Nalgene).

Example 11

15 Solubilization, refolding, and purification of rhTFPI from inclusion bodies using polyphosphate in the absence of chaotropes such as urea (GDS 5327089,92)

About 2 g of rhTFPI (43 ml inclusion body slurry containing 46 mg/ml rhTFPI) was dissolved with mixing in 4 L of 50 mM Tris buffer, pH 10.5 containing 4 g/l polyphosphate (Glass H, FMC Corporation) 2-8°C. Sufficient cysteine and cystine was added to make the solutions 0.1 mM and 0.05 mM respectively. The pH 20 was maintained at pH 10.5 with 1 N NaOH. The refold solution was incubated at 2-8°C with gentle mixing for 72-96 h.

The refold was next adjusted to pH 6 using glacial acetic acid and then filtered through a 0.2 micron filter. An aliquot of the filtered refold was applied to a 200 ml column of SP-Sepharose HP (Pharmacia) previously equilibrated in 0.4 % Glass H, 25 20 mM sodium phosphate pH 6 buffer after loading, the column was washed with 4 column volumes of 0.4 % Glass H, 20 mM sodium phosphate pH6 buffer. The column was eluted using a linear pH gradient from 0.4 % Glass H, 20 mM sodium phosphate buffer pH 6 to 0.4 % Glass H, 50 mM Tris pH 8 buffer. Fractions were collected and analyzed by SDS PAGE. Relatively pure rhTFPI could be refolded and 30 purified in this manner.

Example 12

Improved solubility of rhTFPI in water by formation of a complex between TFPI and polyphosphate (GDS 5327046-47)

About 10 g of purified rhTFPI in about 1 liter of 2 M urea, 125 mM sodium chloride, 20 mM sodium phosphate pH 7.4 buffer was thawed by incubation at 2-8°C for 18-36 h. Sufficient dry urea was added to make the solution 6 M in urea. The solution was then filtered through a 0.2 micron filter. Five g of polyphosphate glass (Glass H, FMC) was dissolved in 50 ml of 6 M urea, adjusted to pH 7 with 1 N NaOH, and added to the protein solution. The solution was then concentrated by ultrafiltration using 1 square foot of membrane (Amicon S1Y3) to about 400 ml (~25 mg/ml) and diafiltered against 10 volumes (about 4 liters) of purified water to remove residual urea. After diafiltration, the solution was concentrated to about 250 ml and removed from the ultrafiltration unit. The ultrafiltration unit was washed with about 150 ml of purified water and the was added to the protein concentrate. The final protein concentrate contained almost 10 g of protein in 400 ml of water (about 24 mg/ml protein). The normal solubility of rhTFPI in water is less than 0.5 mg/ml.

Example 13

Use of cationic polymers for removal of *E. coli* contaminants from TFPI cell lysates and refractile bodies.

The use of cationic polymers to precipitate and remove *E. coli* contaminants from crude TFPI intermediates (lysates, refractile bodies) can significantly improve subsequence process operations (refolding, chromatography etc.) A random screening of cationic polymers identified candidates which selectively precipitate bacterial contaminants while TFPI remains in solution. Specifically, Betz polymer 624 precipitated substantial amounts of bacterial contaminants, while leaving TFPI in solution in an aqueous environment.

Solubilized TFPI refractile bodies (in 3.5 M guanidine hydrochloride, 2 M sodium chloride, 50 mM TRIS, 50 mM dithiothreitol, pH7.1) was the starting material used for a polymer screening experiment. This material was diluted 10 fold into a 0.5% solution of various polymers. The precipitates from this experiment

were analyzed by SDS-PAGE for the presence of TFPI. Betz polymer 624 precipitated substantial amounts of contaminants, no TFPI, and resulted in a clear aqueous solution.

Example 14

5 The use of aqueous two phase extraction with a polyethylene glycol (PEG), polyphosphate, urea system offers processing advantages for TFPI purification. Typical aqueous two phase systems consist of two polymer systems (e.g., PEG and dextran) or a polymer and salt (e.g., PEG and sulfate). The system described here has advantages in that the polyphosphate chain length can be optimized for the
10 separation, is inexpensive and is specific in removing problematic contaminants from TFPI refractile bodies known to interfere with refolding and chromatography (native polyphosphate and associated divalent metals).

15 TFPI refractile bodies were solubilized in 7 M urea, 10 mM CAPS, 1% monothioglycerol pH10. Polyphosphate and PEG of different chain lengths were added to form two phases. Upon phases separation, the TFPI partitioned into the PEG rich upper phase, leaving the polyphosphates and associated contaminants in the lower phase. Separation is effected by both PEG and polyphosphate chain length and can be optimized by varying both.

Example 15

20 Charged polymer facilitated refolding of recombinant tissue plasminogen activator (t-PA) from *E. coli* inclusion bodies

25 Five grams (wet weight) of inclusion bodies containing about 2 grams of recombinant tissue plasminogen activator are added to about 1 liter of 0.5 % Glass H, 50 mM Tris buffer pH 10.8 containing 1 mM reduced glutathione (GSH) and 0.2 mM glutthione disulfide (GSSG). The mixture is thoroughly blended using a polytron (Brinkman) homogenizer for 2-3 minutes to thoroughly disperse the inclusion bodies. The mixture is incubated with mixing using an overhead stirrer for 15 minutes while the pH is maintained at 10.5-10.9 using 1 N NaOH. The mixture is then gently mixed for 48-72 hours at 2-8oC.

30 Example 16

- 45 -

Charged polymer facilitated refolding of bovine somatotropin from *E. coli* inclusion bodies

Ten grams (wet weight) of inclusion bodies containing 5 grams of bovine somatotropin are added to about 1 liter of 1% Glass H, 50 mM Tris buffer pH 10.5.

5 The mixture is thoroughly blended using a polytron (Brinkman) homogenizer for 2-3 minutes to thoroughly disperse the inclusion bodies. The mixture is incubated with mixing using an overhead stirrer for 15 minutes while the pH is maintained at 10.4-10.6 using 1 N NaOH. Solid cysteine (121 mg) is added to make the reaction 1 mM cysteine, and the refolding reaction is mixed for 48-72 hours

10 The patents, patent applications and publications cited herein are incorporated by reference.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from 15 the spirit and the scope of the appended claims.

We Claim:

1. An aqueous formulation comprising TFPI and a charged polymer wherein the concentration of TFPI is greater than 1 mg/ml.
- 5 2. The aqueous formulation of claim 1 wherein the concentration of TFPI is greater than 5 mg/ml.
3. The aqueous formulation of claim 1 wherein the concentration of TFPI is greater than 10 mg/ml.
4. The aqueous formulation of claim 1 wherein the concentration of TFPI is greater than 20 mg/ml.
- 10 5. The aqueous formulation of claim 1 which is pharmaceutically acceptable.
6. The aqueous formulation of claim 1 wherein the charged polymer is a sulfated polysaccharide.
7. The aqueous formulation of claim 1 wherein the charged polymer is heparin.
8. The aqueous formulation of claim 1 wherein the charged polymer is dextran sulfate.
- 15 9. The aqueous formulation of claim 1 wherein the charged polymer is polyphosphate.
10. A method of modifying the solubility of a protein having a first domain which has a net positive charge and a second domain which has a net negative charge, comprising the steps of:
20 adding to the protein an aqueous solution of a charged polymer to reduce intermolecular or intramolecular interactions between the positively and negatively charged domains.
11. The method of claim 10 wherein the first domain has a charge density of at least five cationic amino acids in a series of ten consecutive amino acids.
- 25 12. The method of claim 10 wherein the first domain comprises five consecutive cationic amino acids.
13. The method of claim 10 wherein the second domain comprises five consecutive anionic amino acids.

14. The method of claim 10 wherein the second domain comprises five anionic amino acids in a series of ten consecutive amino acids.
15. The method of claim 10 wherein the protein is TFPI.
16. The method of claim 10 wherein the protein is a TFPI mutein.
- 5 17. The method of claim 10 wherein the protein is TFPI-2.
18. The method of claim 10 wherein the protein is in an insoluble form prior to the step of adding.
19. The method of claim 10 wherein a chaotropic agent is also added to the protein.
20. The method of claim 10 wherein the specific activity of the protein is increased
10 by said step of adding.
21. The method of claim 10 wherein the charged polymer is immobilized on a solid support.
22. The method of claim 10 further comprising:
applying the protein to a solid support before adding the charged polymer.
- 15 23. The method of claim 10 further comprising:
applying the protein to a solid support after adding the charged polymer.
24. The method of claim 22 wherein the solid support is an ion exchange resin.
25. The method of claim 23 wherein the solid support is an ion exchange resin.
26. The method of claim 20 wherein the protein is TFPI.
- 20 27. The method of claim 24 wherein the resin and the polymer have opposite net charges.
28. The method of claim 25 wherein the resin and the polymer have opposite net charges.
29. The method of claim 24 wherein the resin and the polymer have the same net
25 charge.
30. The method of claim 25 wherein the resin and the polymer have the same net charge.
31. The method of claim 24 wherein the charged polymer is added in a concentration gradient to effect selective elution from the solid support.

32. A method of refolding an improperly folded or denatured protein comprising the step of adding charged polymers to a solution comprising said protein prior to allowing said protein to refold.
33. The method of claim 32, wherein said polymer is a sulfated polysaccharide.
- 5 34. The method of claim 33, wherein said sulfated polysaccharide is dextran sulfate.
35. The method of claim 33, wherein said sulfated polysaccharide is heparin.
36. A method of refolding TFPI comprising the step of adding a charged polymer to a solution comprising improperly folded or denatured TFPI prior to allowing said TFPI to refold.
- 10 37. The method of claim 36, wherein the polymer is dextran sulfate.
38. The method of claim 36, wherein the polymer is heparin.
39. The method according to claim 38, wherein the heparin is added in solution.
40. The method according to claim 36 further comprising the steps of:
 - incubating said solution to allow said TFPI to refold, adding salt to
- 15 disassociate the polymer from the TFPI, passing the solution over an HIC column, and recovering the TFPI.
41. A method of refolding TFPI comprising the step of immobilizing polymers of sulfated polysaccharides on a column and passing a solution of denatured TFPI through the column and eluting the refolded TFPI after the refolding has occurred.
- 20 42. The method of claim 41, wherein the sulfated polysaccharide is dextran sulfate.
43. The method of claim 41, wherein the sulfated polysaccharide is heparin.
44. A pharmaceutically acceptable composition comprising more than 0.2 mg/mL TFPI and a solubilizing agent, said solubilizing agent selected from the group consisting of: (a) acetate ion; (b) sodium chloride; (c) citrate ion; (d) isocitrate ion;
- 25 (e) glycine; (f) glutamate; (g) succinate ion; (h) histidine; (i) imidazole; and (j) SDS.
45. The composition of claim 44 wherein TFPI is present at a concentration of at least 1 mg/mL.
46. The composition of claim 44 wherein TFPI is present at a concentration of at
- 30 least 10 mg/mL.

47. The composition of claim 44 further comprising a secondary solubilizer, said secondary solubilizer selected from the group consisting of:
 - (a)polyethylene glycol; (b)sucrose; (c)mannitol; and (d)sorbitol.
48. The composition of claim 44 further comprising sodium phosphate at a concentration greater than 20mM.
 - 5 49. The composition of claim 44, wherein the composition is hypertonic.
 50. The composition of claim 49 wherein the hypertonic composition comprises 0.5M NaCl.
 51. The composition of claim 49 wherein the hypertonic composition comprises
 - 10 0.5M NaPO₄.
 52. The composition of claim 49 wherein the hypertonic composition comprises 0.5M sodium citrate.
 53. The composition of claim 49 wherein the hypertonic composition comprises 0.5M sodium isocitrate.
 - 15 54. The composition of claim 44 wherein the composition is isotonic.
 55. The composition of claim 44 wherein the pH of the composition is below pH 7.0 and the solubilizer is not glycine.
 56. The composition of claim 55 wherein the pH of the composition is pH 4.5 or below.
 - 20 57. The composition of claim 44 wherein the solubilizer is acetate ion and the acetate ion is present in the composition as sodium acetate or potassium acetate at a concentration from 5 mM to 20 mM.
 58. The composition of claim 44 wherein the solubilizer is sodium chloride and the sodium chloride is present in the composition at a concentration of at least 0.5M.
 - 25 59. The composition of claim 44 wherein the solubilizer is citrate ion and the citrate ion is present in the composition as sodium citrate or potassium citrate at a concentration from 100 mM to 500 mM.
 60. The composition of claim 44 wherein the solubilizer is isocitrate ion and the isocitrate ion is present in the composition as sodium isocitrate or potassium isocitrate
 - 30 at a concentration from 100 mM to 500 mM.

61. The composition of claim 44 wherein the solubilizer is glycine and the glycine is present in the composition at a concentration from 5 mM to 20 mM.
62. The composition of claim 44 wherein the solubilizer is glutamate and the glutamate is present in the composition at a concentration from 5 mM to 20 mM.
- 5 63. The composition of claim 44 wherein the solubilizer is succinate ion and the succinate ion is present in the composition as sodium succinate at a concentration from 5 mM to 20 mM.
64. The composition of claim 44 wherein the solubilizer is histidine and the histidine is present in the composition at a concentration from 5 mM to 20 mM.
- 10 65. The composition of claim 44 wherein the solubilizer is imidazole and the imidazole is present in the composition at a concentration from 5 mM to 20 mM.
66. The composition of claim 44 wherein the solubilizer is sodium docecy1 sulfate and the sodium docecy1 sulfate is present in the composition at a concentration of 0.001 % to 0.1 % (weight / volume).
- 15 67. The composition of claim 47 wherein the secondary solubilizer is polyethylene glycol and the polyethylene glycol is present in the composition at a concentration of 0.2 % to 10 % (weight / volume).
68. The composition of claim 47 wherein the secondary solubilizer is sucrose and the sucrose is present in the composition at a concentration of 0.2 % to 10 % (weight / volume).
- 20 69. The composition of claim 47 wherein the secondary solubilizer is mannitol and the mannitol is present in the composition at a concentration of 1.0 % to 5.0 % (weight / volume).
70. The composition of claim 47 wherein the secondary solubilizer is sorbitol and the sorbitol is present in the composition at a concentration of 0.2 % to 10 % (weight / volume).
- 25

1 / 34

TFPI Peak Fractions from
Load HIC

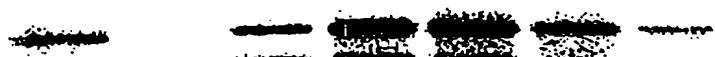


FIGURE 1

2 / 34

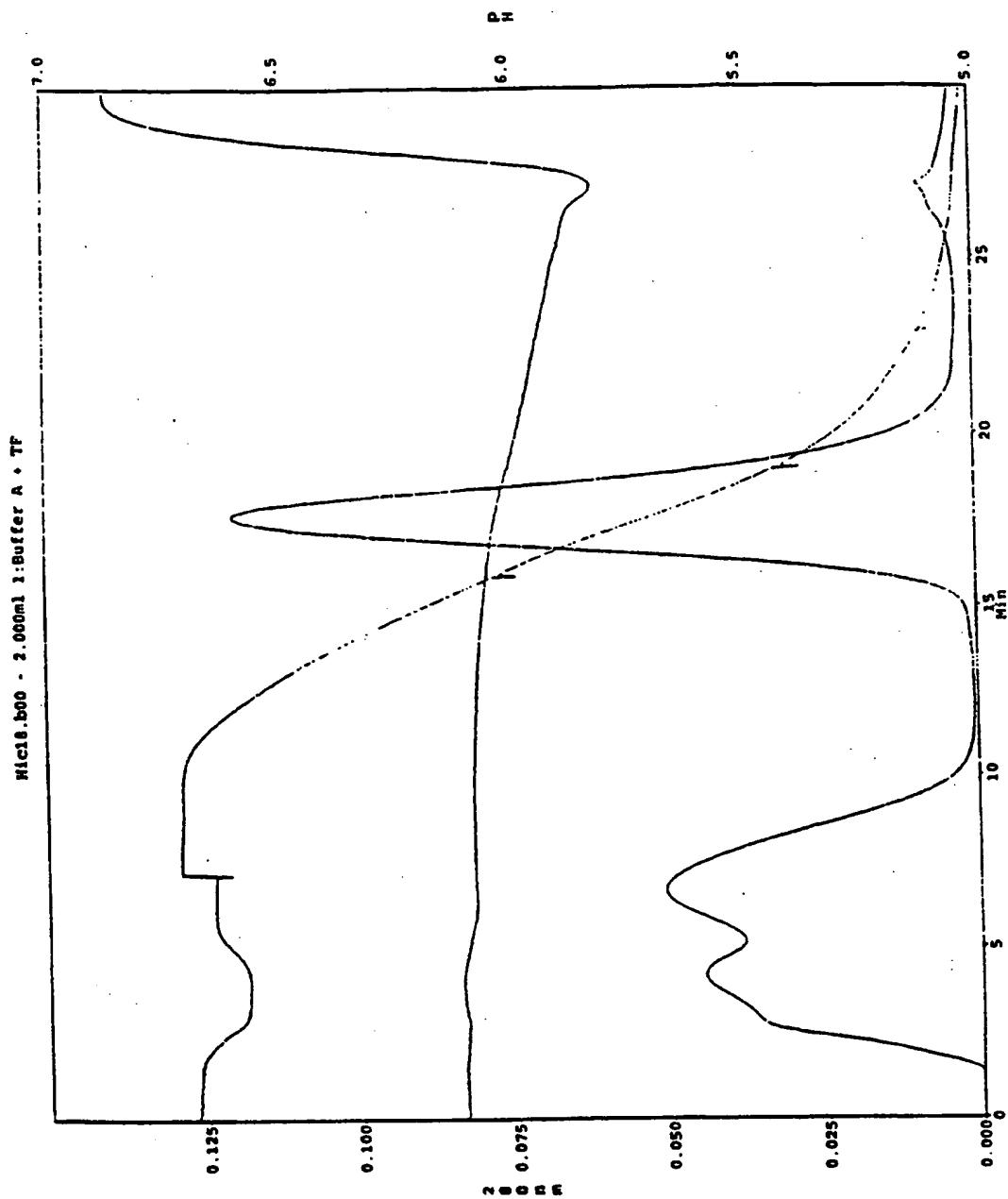


FIGURE 2

3 / 34

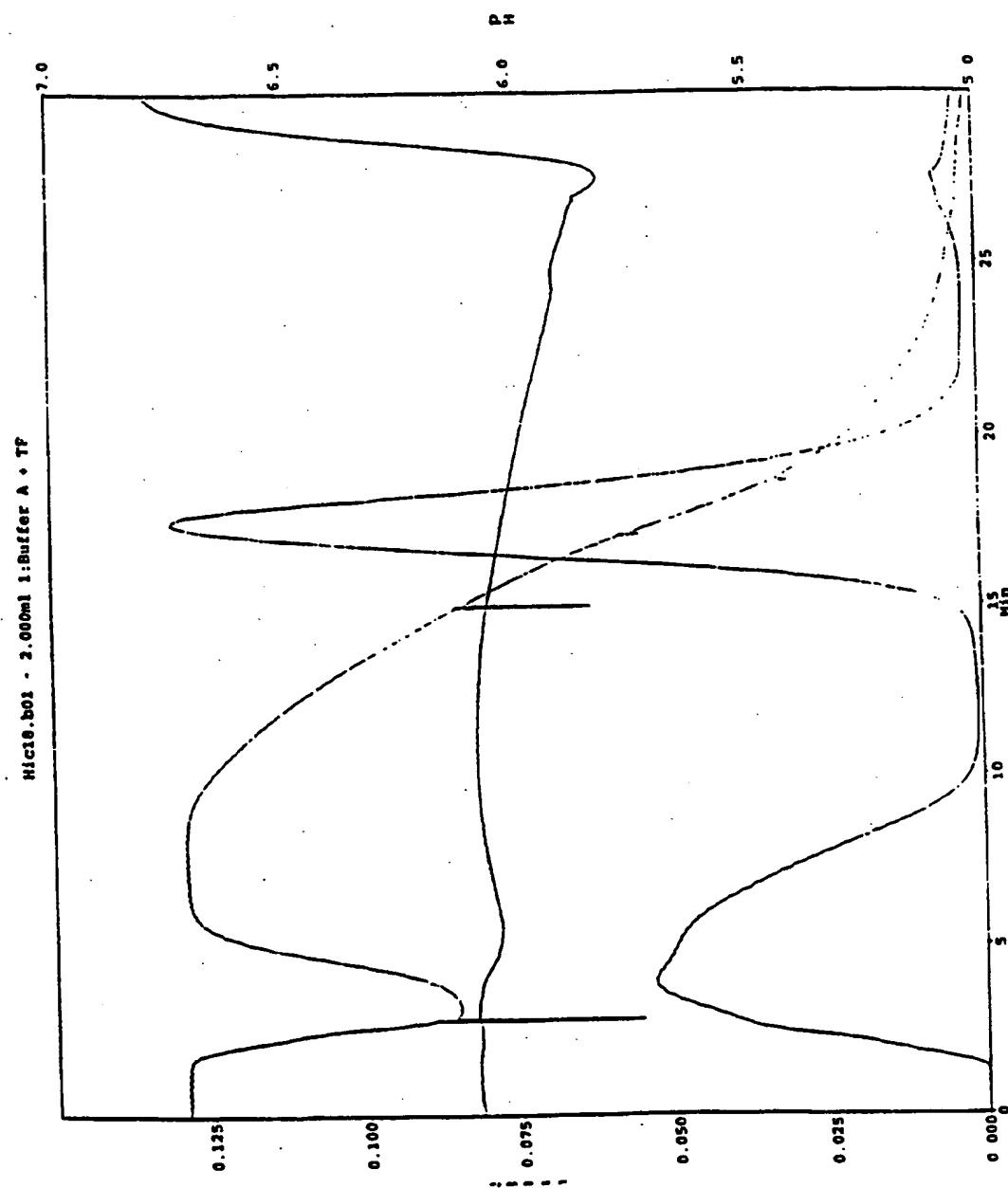


FIGURE 3

4 / 34

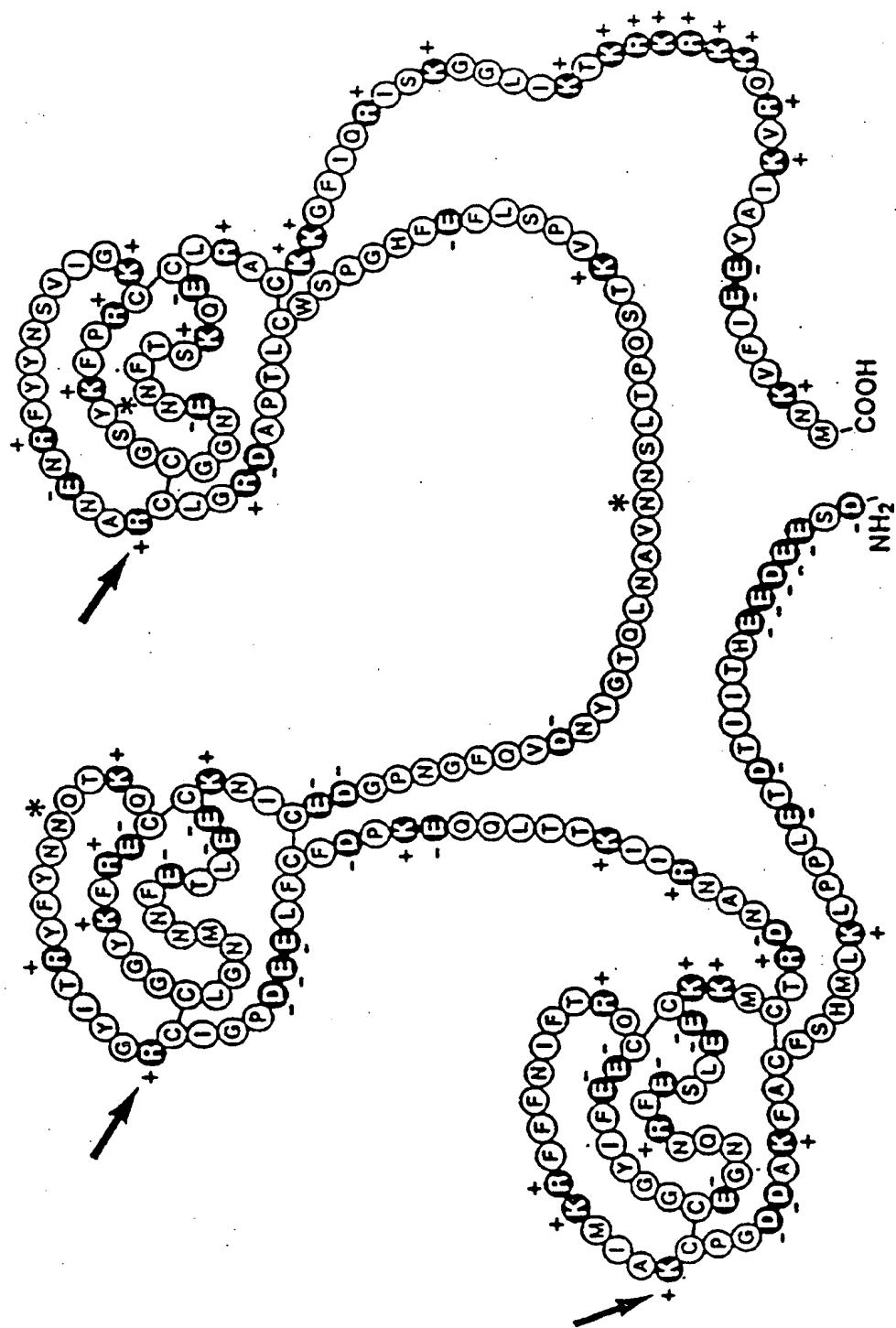


FIGURE 4

5 / 34

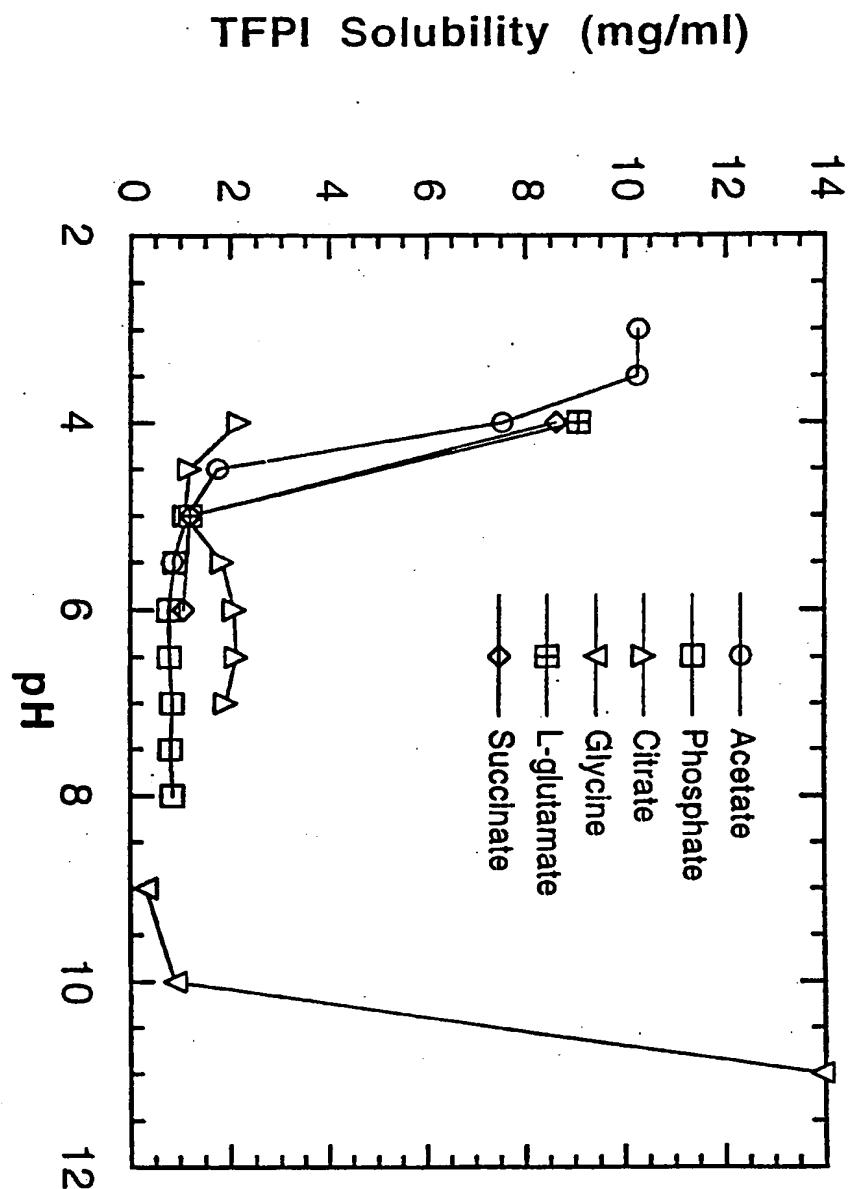
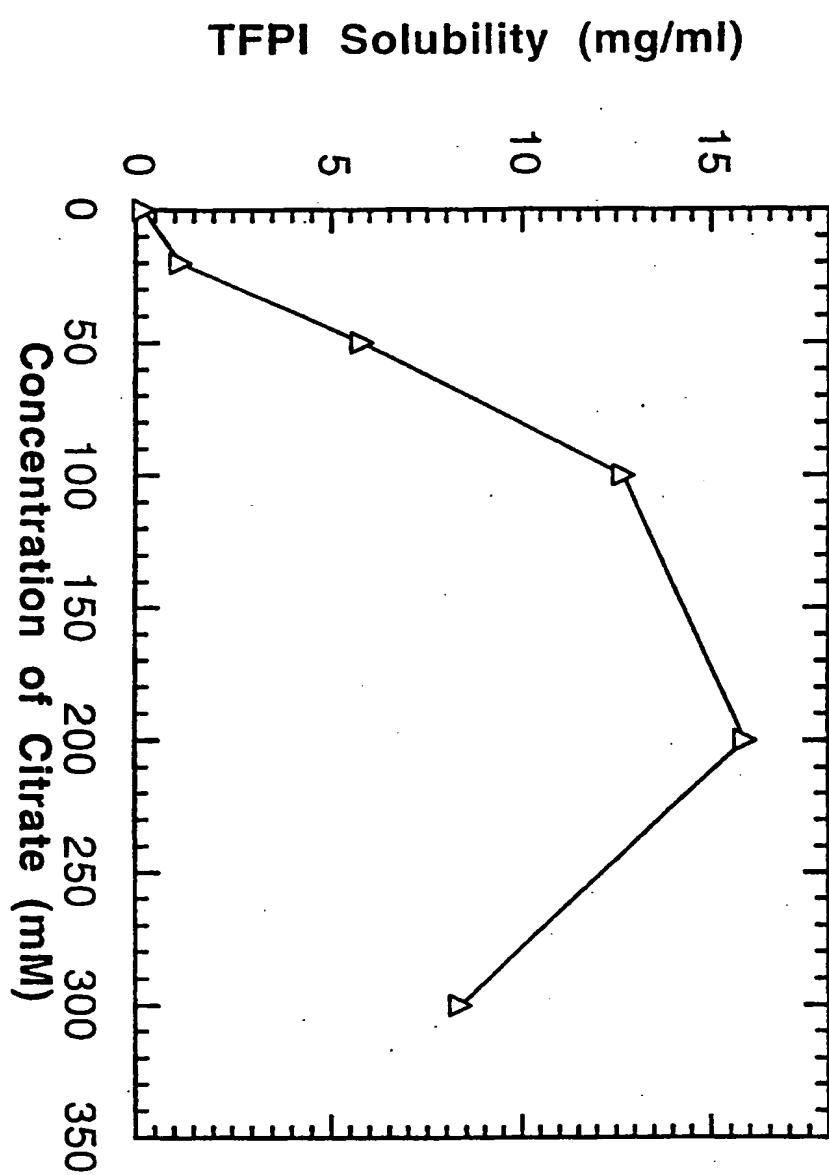


FIGURE 5

6 / 34

**FIGURE 6**

7 / 34

TFPI Solubility (mg/ml)

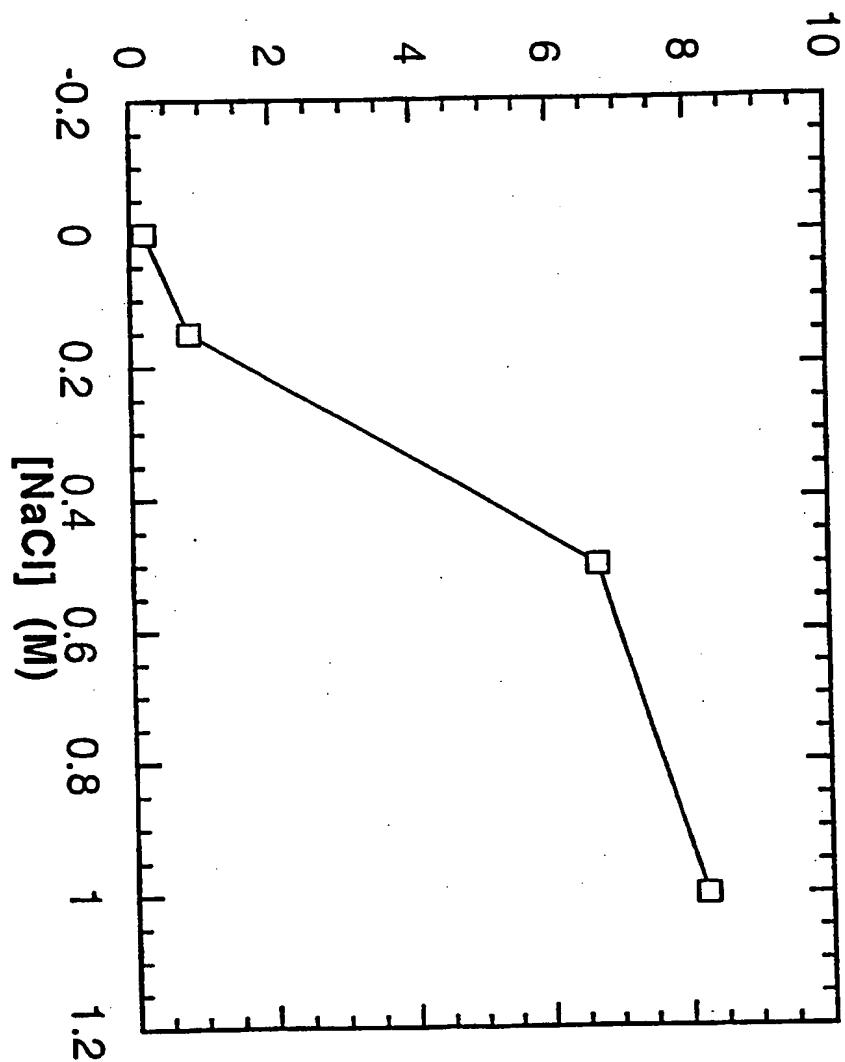


FIGURE 7

8 / 34

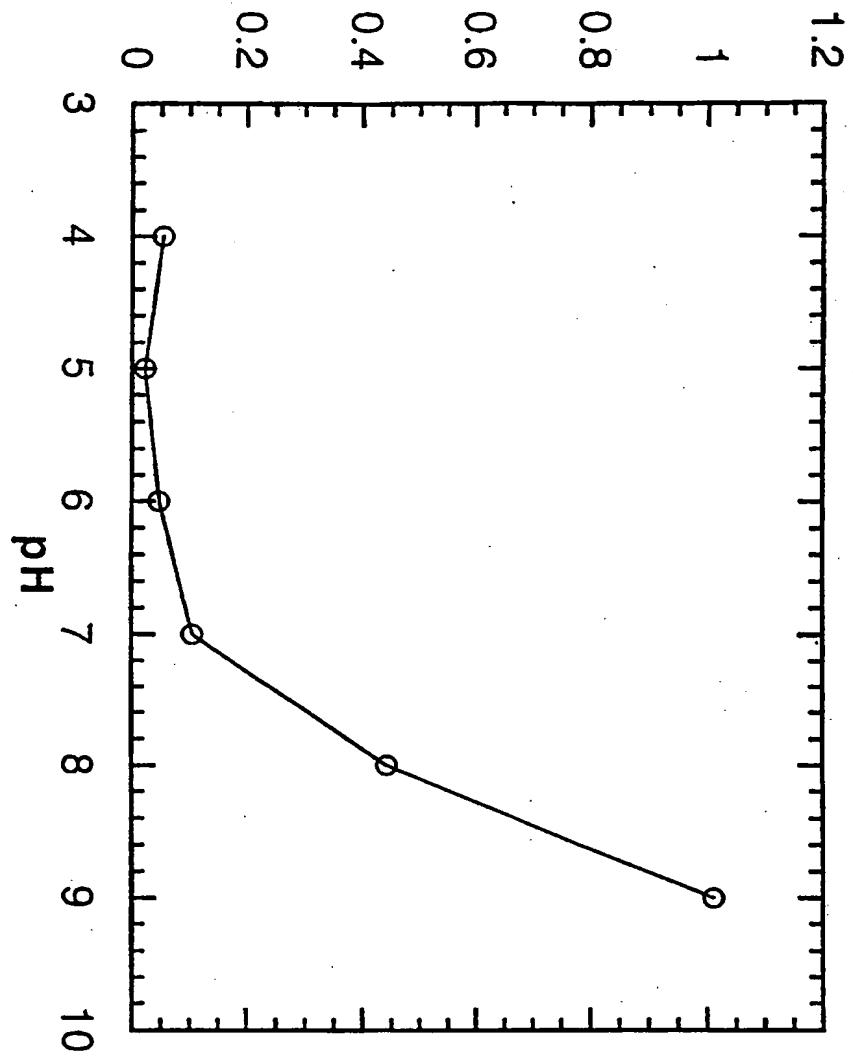
Decay Rate Constant (day⁻¹)

FIGURE 8

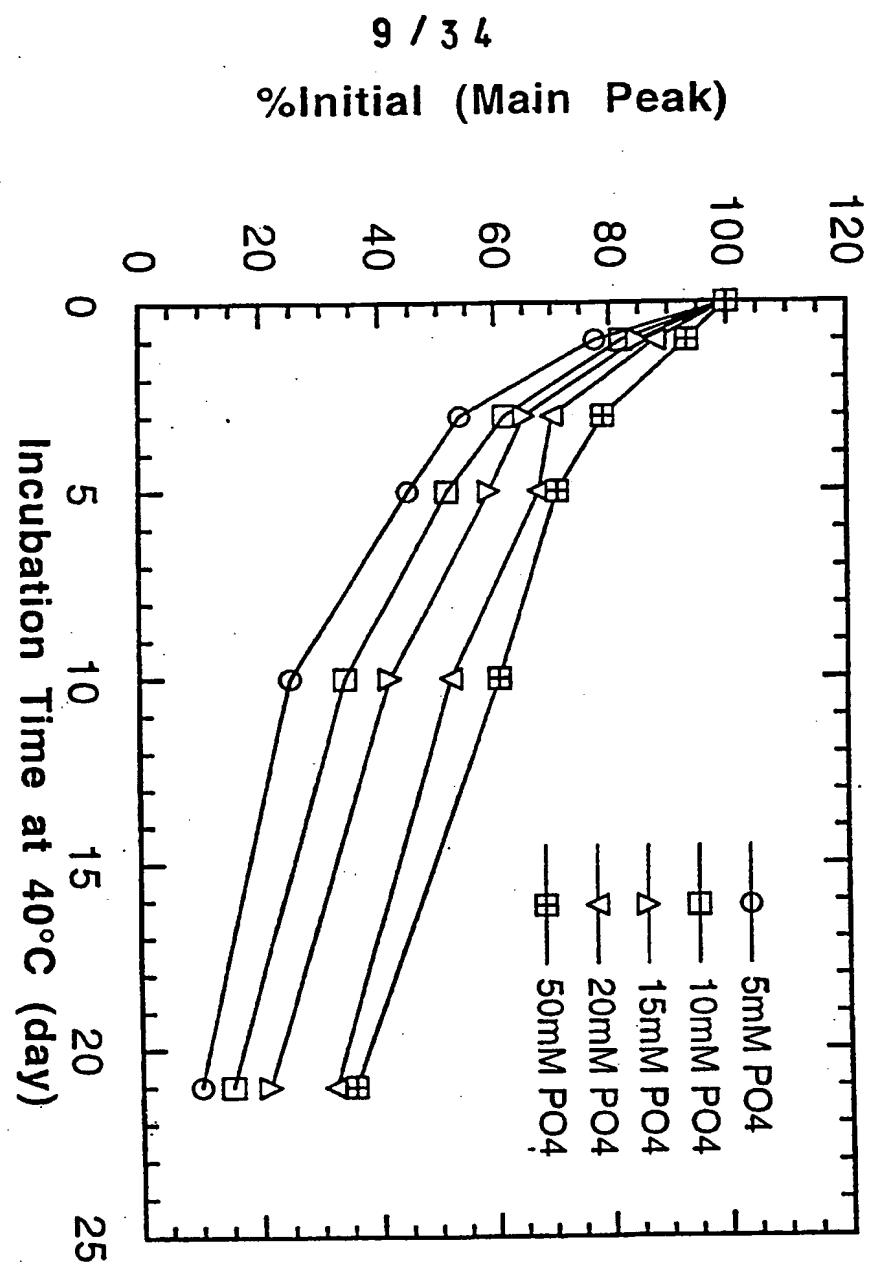


FIGURE 9A

10 / 34

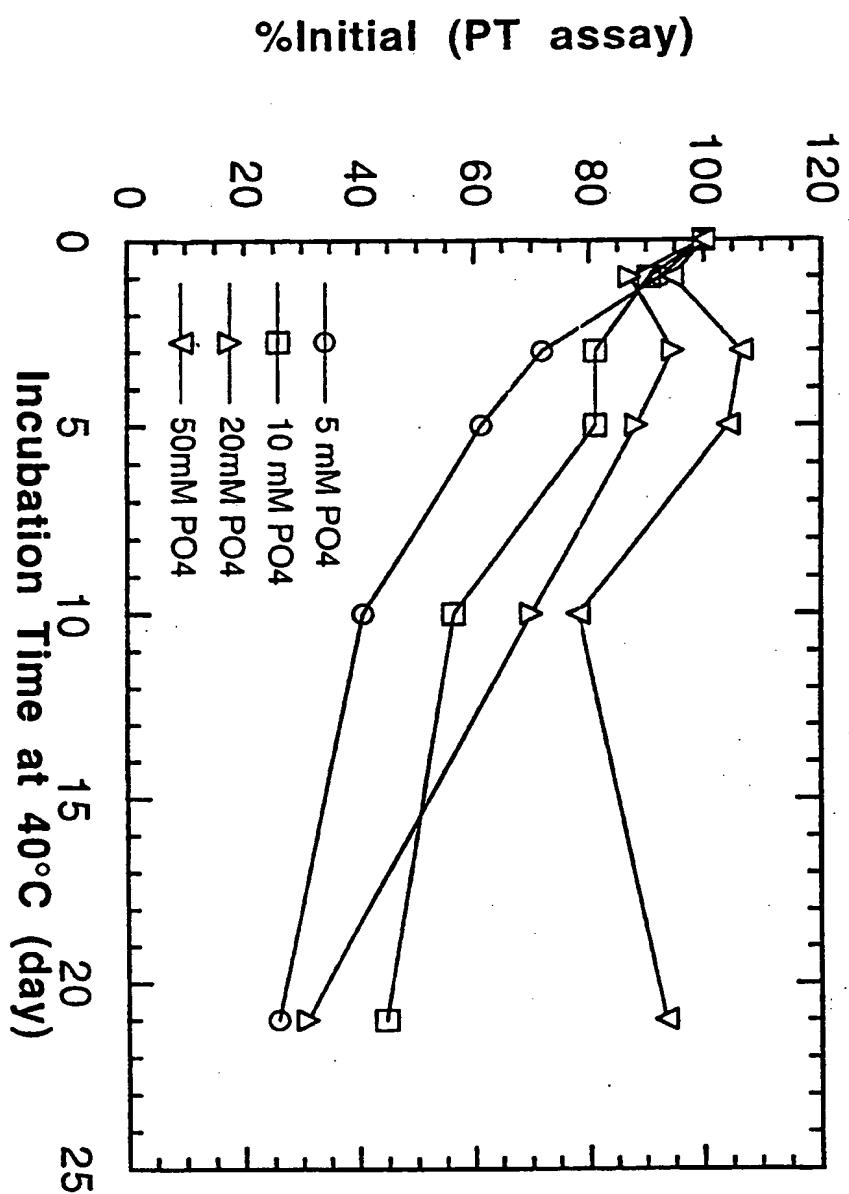


FIGURE 9B

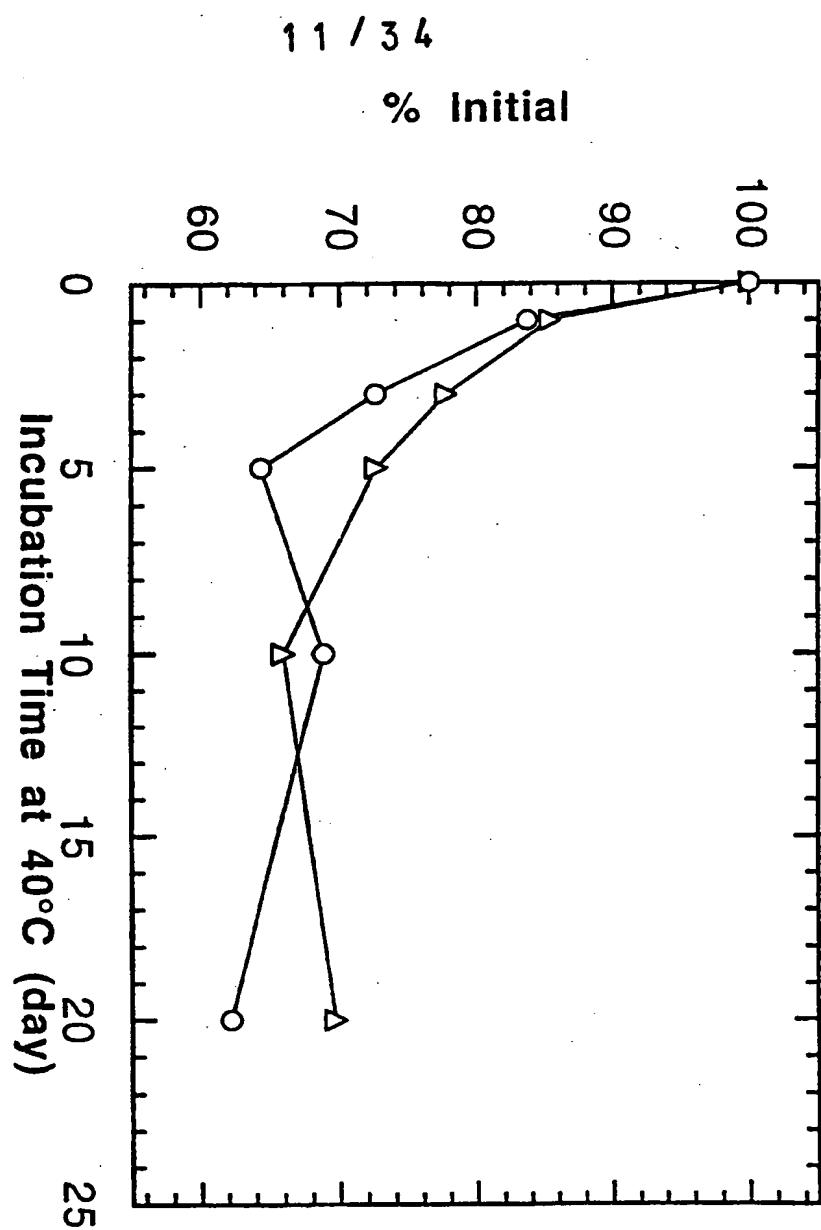


FIGURE 10

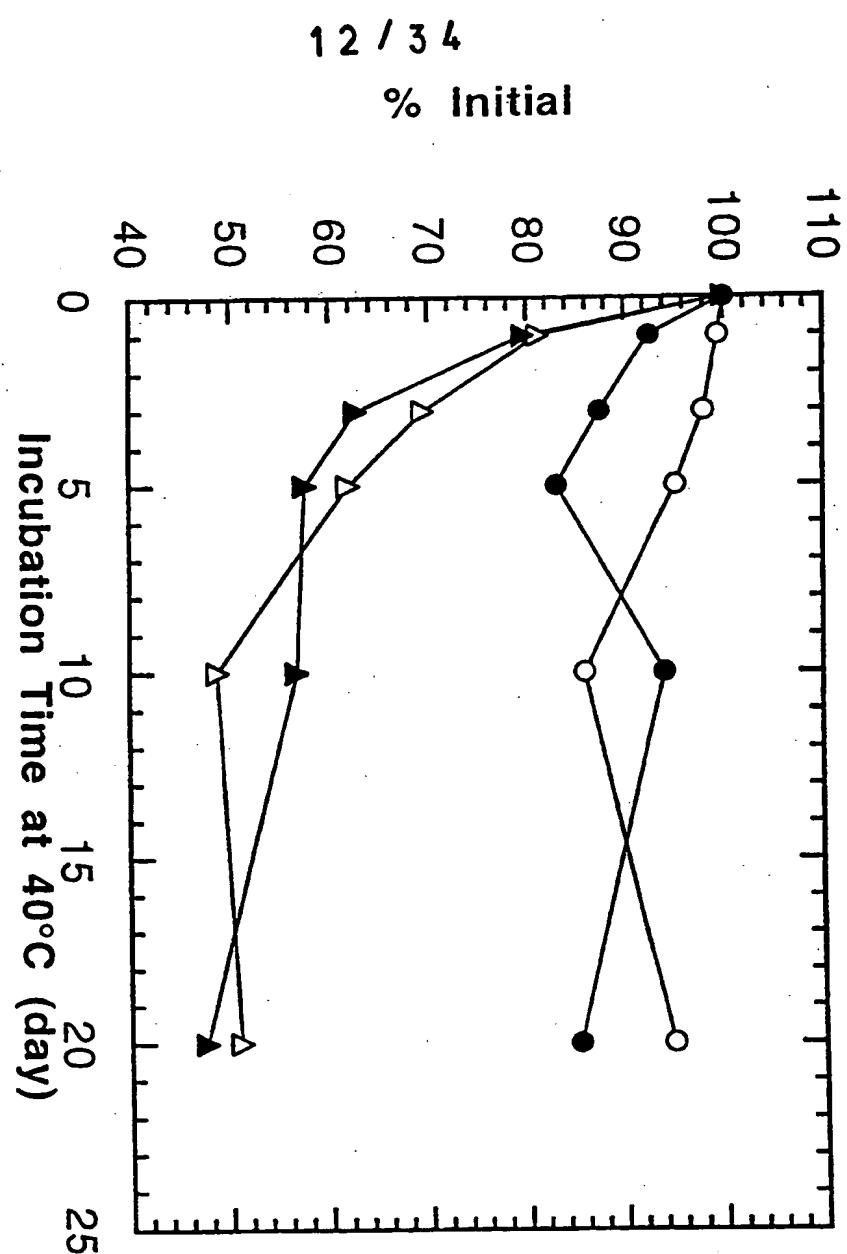


FIGURE 11

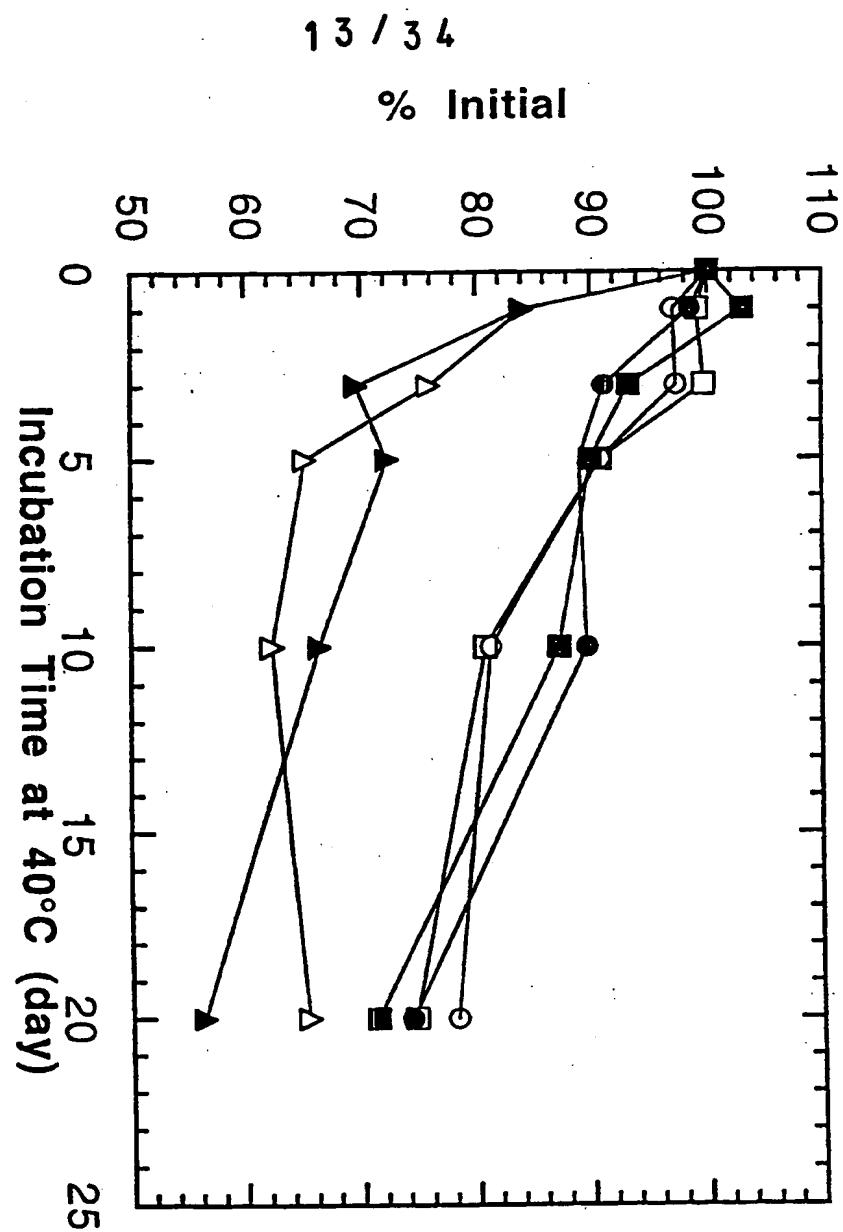


FIGURE 12

14 / 34

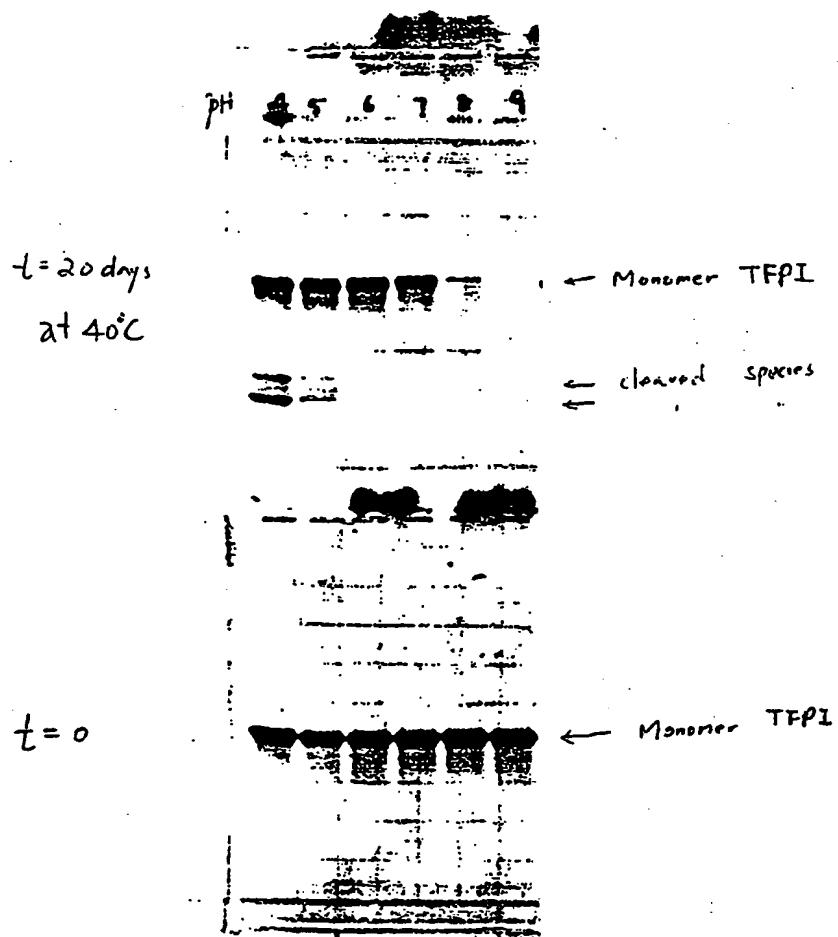
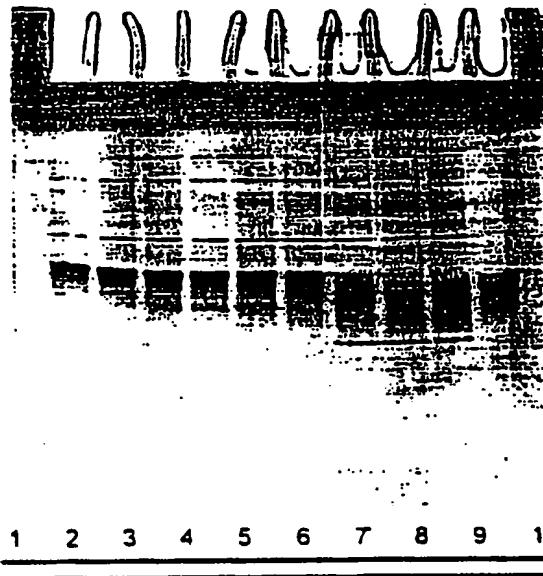


FIGURE 13

15 / 34

Non-reducing SDS-PAGE analysis of polyphosphate refold timepoints.

Lane #	Lane Description
1	Dissolved refractile bodies
2	T ₀ hour
3	T ₁₈ hour
4	T ₂₁ hour
5	T ₂₅ hour
6	T ₄₃ hour
7	T ₆₈ hour
8	T ₉₀ hour
9	T ₉₆ hour
10	SC-59735

1	Dissolved refractile bodies
2	T ₀ hour
3	T ₁₈ hour
4	T ₂₁ hour
5	T ₂₅ hour
6	T ₄₃ hour
7	T ₆₈ hour
8	T ₉₀ hour
9	T ₉₆ hour
10	SC-59735

FIGURE 14

16 / 34

Sepharose gradient elution of polyphosphate refold from run #
41295.

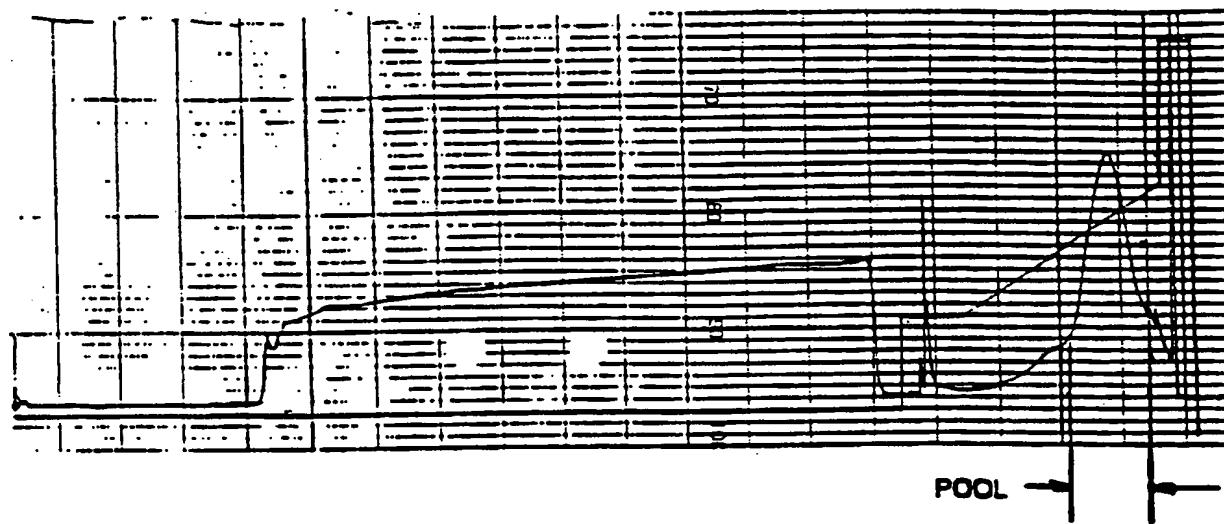
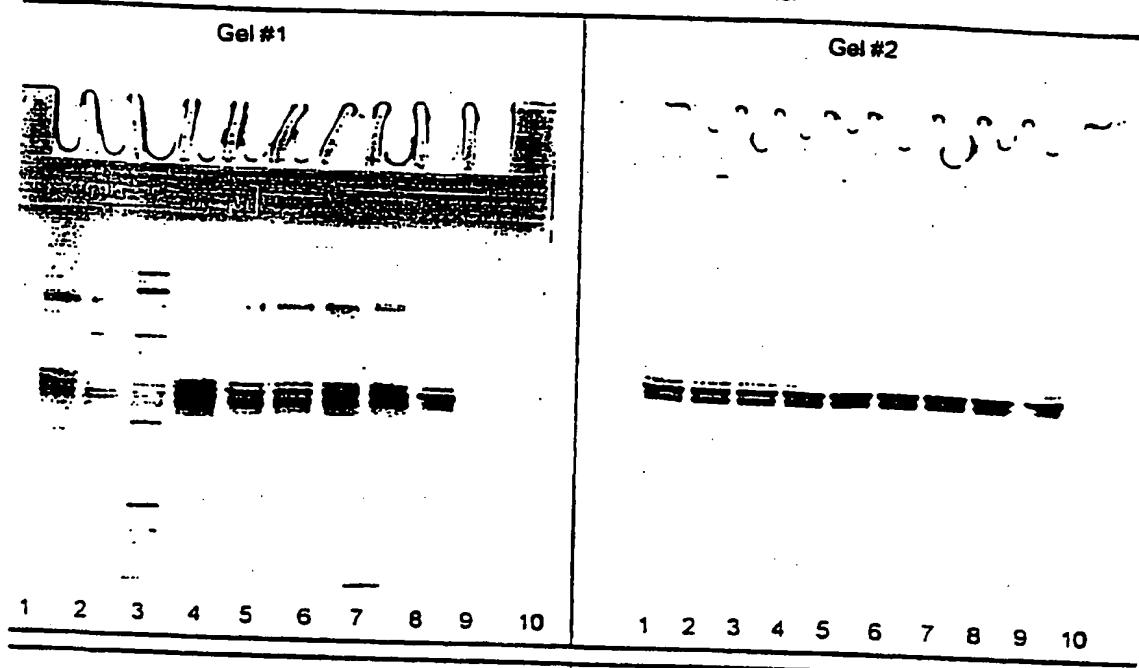


FIGURE 15

17 / 34

Non-reducing SDS-PAGE analysis of SP-Sepharose fractions.



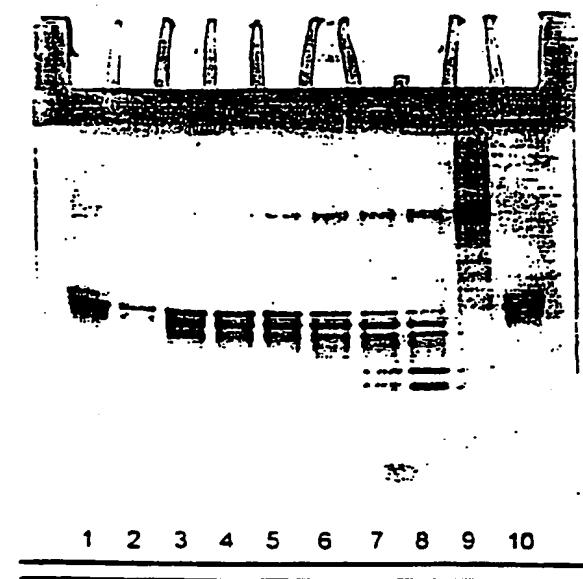
Lane #	Lane Description
1	Load
2	Flow through and wash
3	0.3 M wash
4	Pool before fractions
5	Fraction 1
6	Fraction 3
7	Fraction 5
8	Fraction 7
9	SC-59735
10	

Lane #	Lane Description
1	Fraction 9
2	Fraction 11
3	Fraction 13
4	Fraction 15
5	Fraction 17
6	Fraction 19
7	Fraction 21
8	Fraction 23
9	SC-59735
10	

FIGURE 16 A

18 / 34

Non-reducing SDS-PAGE analysis of SP-Sepharose fractions.



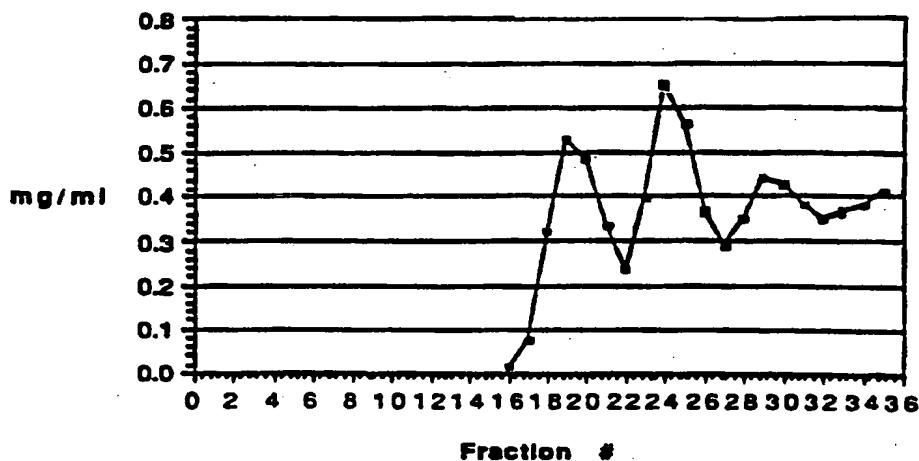
Lane #	Lane Description
1	Fraction 25
2	Fraction 27
3	Fraction 29
4	Fraction 31
5	Fraction 33
6	Fraction 35
7	Fraction 37
8	Fraction 39
9	1.0M elution
10	SC-59735

Lane #	Lane Description
1	Fraction 25
2	Fraction 27
3	Fraction 29
4	Fraction 31
5	Fraction 33
6	Fraction 35
7	Fraction 37
8	Fraction 39
9	1.0M elution
10	SC-59735

FIGURE 16B

19 / 34

Q-Sepharose elution profile for polyphosphat. a refold.



Aggregate content of Q-Sepharose fractions from 100 liter polyphosphate refold.

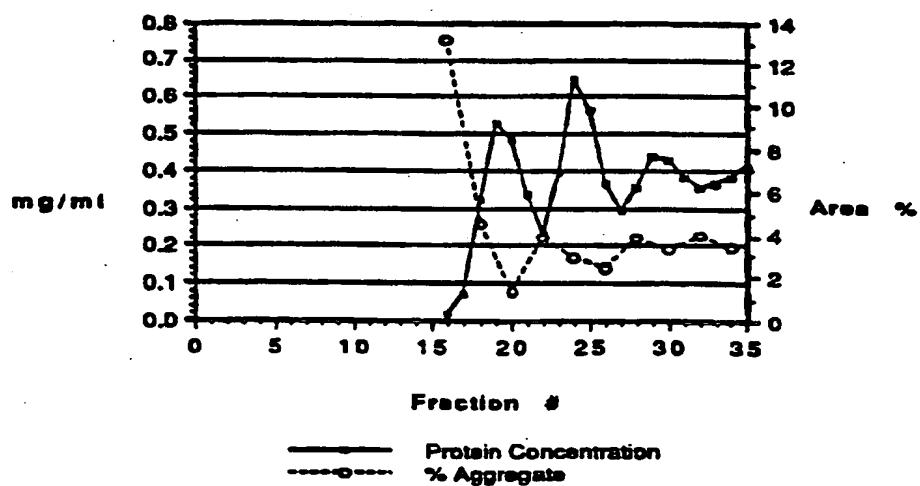


FIGURE 17

20 / 34

Non-reducing SDS-PAGE analysis of Q-Sepharose fractions.

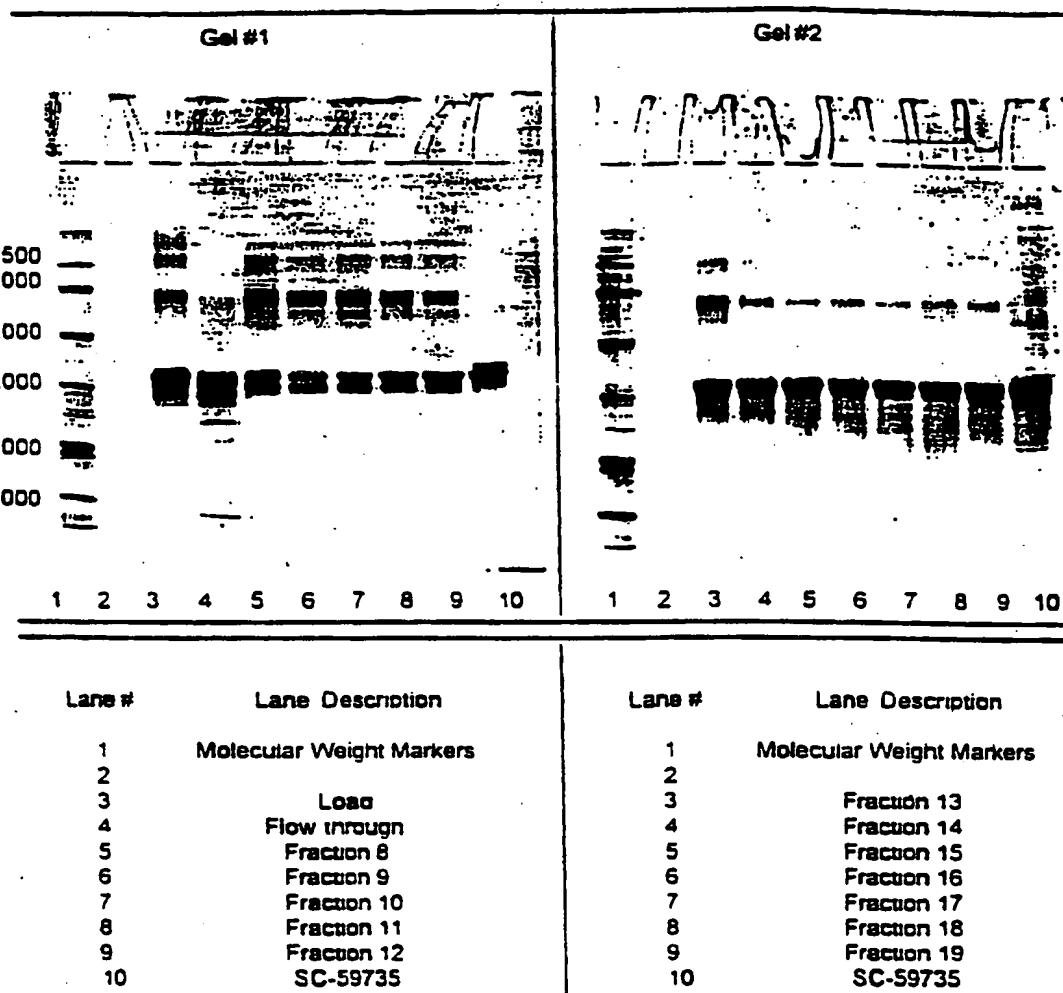
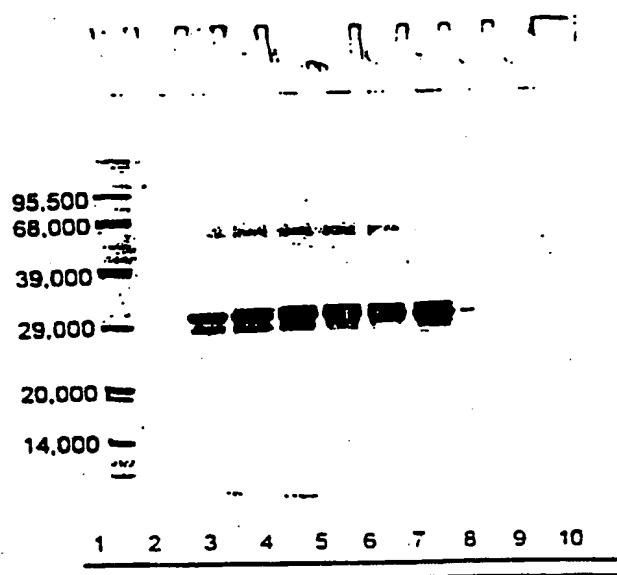


FIGURE 18A

21 / 34

Non-reducing SDS-PAGE analysis of Q-Sepharose fractions.



Lane # Lane Description

1	Molecular Weight Markers
2	
3	Fraction 20
4	Fraction 21
5	Fraction 22
6	Fraction 23
7	Fraction 24
8	SC-59735
9	
10	

FIGURE 18B

22 / 34

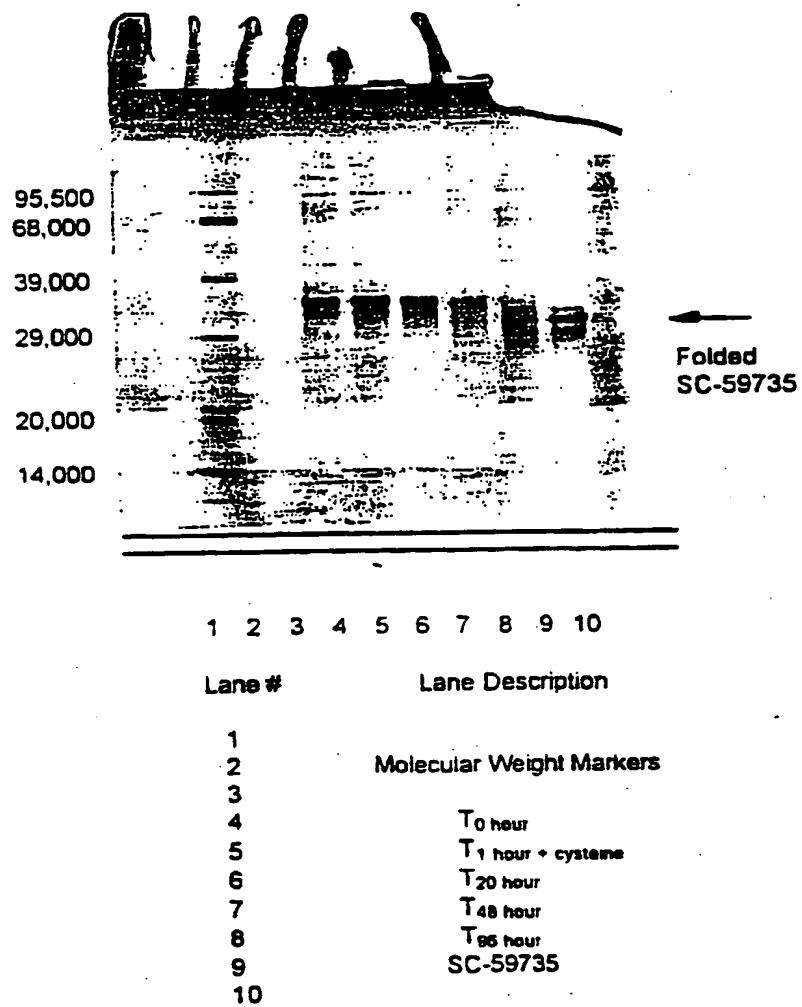
Non-reducing SDS-PAGE analysis PEI refold timepoints.

FIGURE 19

23 / 34

Sp-Sepharose gradient elution of PEI refold from run # 195005.

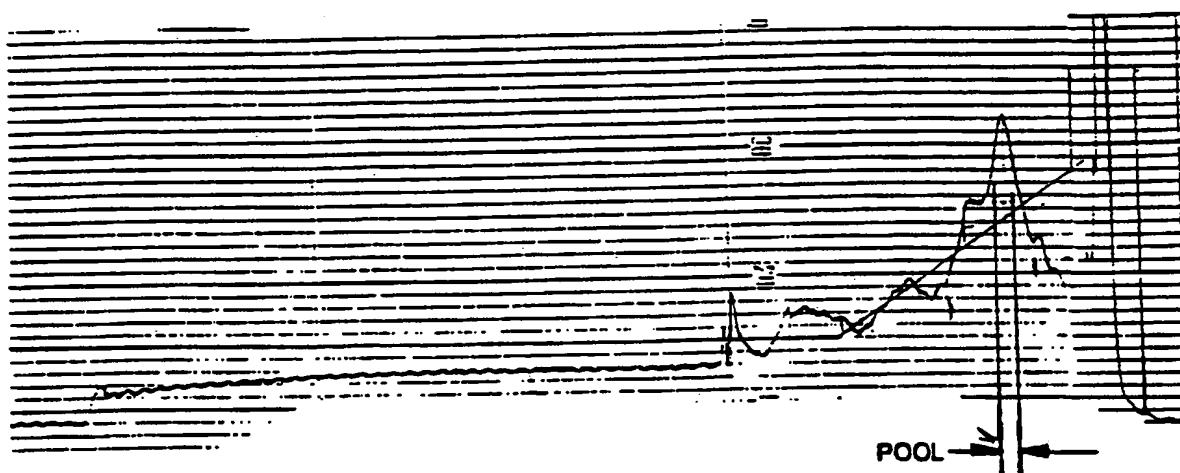
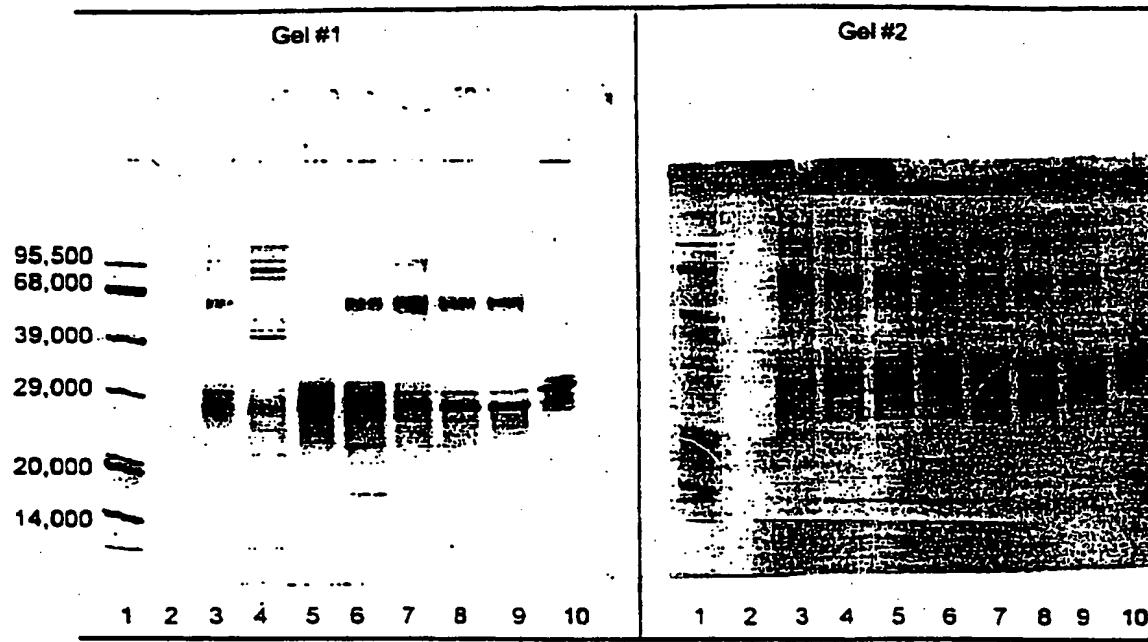


FIGURE 20

24 / 34

Non-reducing SDS-PAGE analysis of SP-Sepharose fractions.



Lane

Lane Description

1	Molecular Weight Markers
2	
3	Load
4	Flow through
5	Wash
6	Pool before fractions
7	Fraction 1
8	Fraction 5
9	Fraction 10
10	SC-59735

Lane

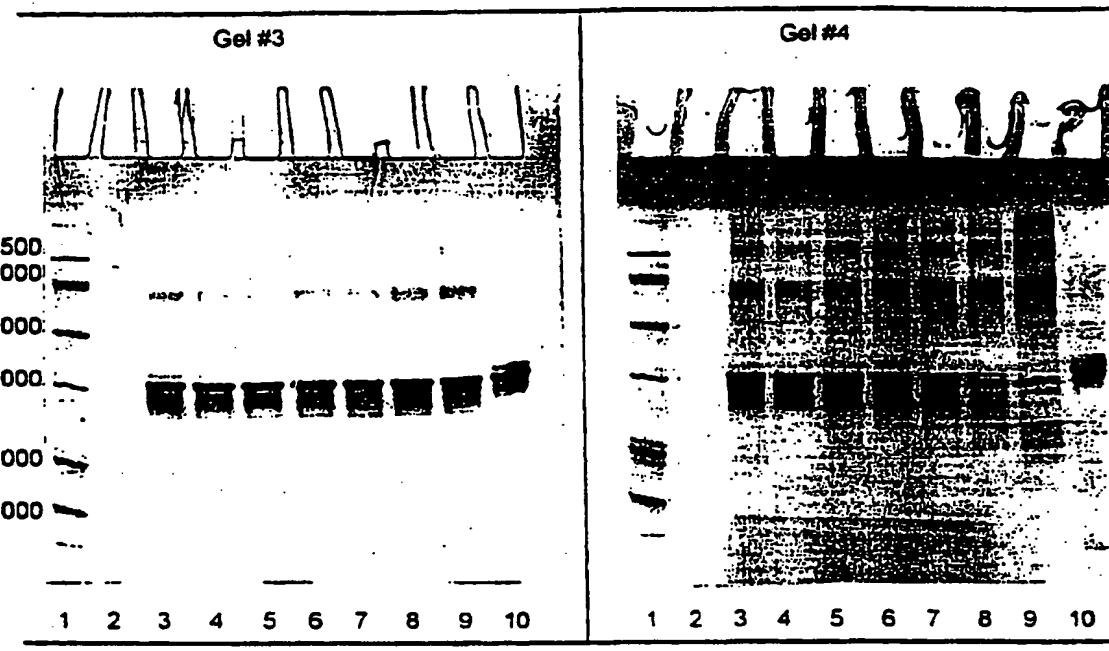
Lane Description

1	Molecular Weight Markers
2	
3	Fraction 15
4	Fraction 20
5	Fraction 21
6	Fraction 22
7	Fraction 23
8	Fraction 24
9	Fraction 25
10	SC-59735

FIGURE 21A

25 / 34

Non-reducing SDS-PAGE analysis of SP-Sephadex fractions.



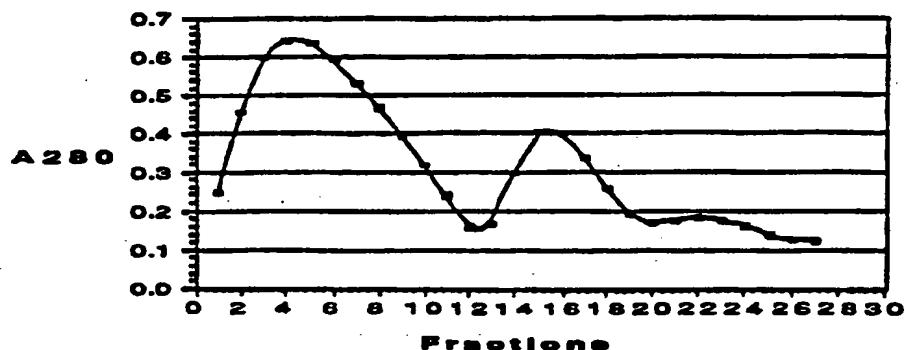
Lane #	Lane Description	Lane #	Lane Description
1	Molecular Weight Markers	1	Molecular Weight Markers
2		2	
3	Fraction 26	3	Fraction 33
4	Fraction 27	4	Fraction 34
5	Fraction 28	5	Fraction 35
6	Fraction 29	6	Fraction 36
7	Fraction 30	7	Fraction 38
8	Fraction 31	8	Fraction 40
9	Fraction 32	9	Pool after fractions
10	SC-59735	10	SC-59735

FIGURE 21B

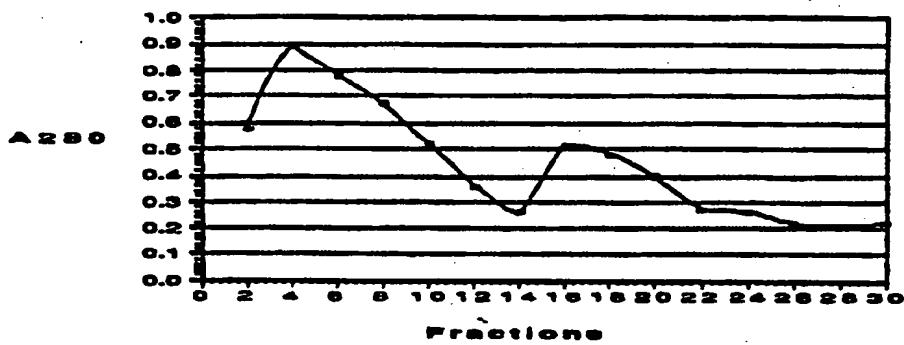
26 / 34

Protein Concentration Profiles of Q Sepharose Elution.

195004



195005



195006

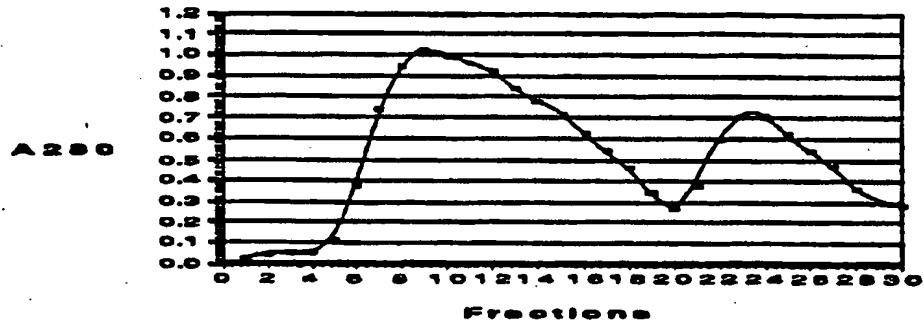
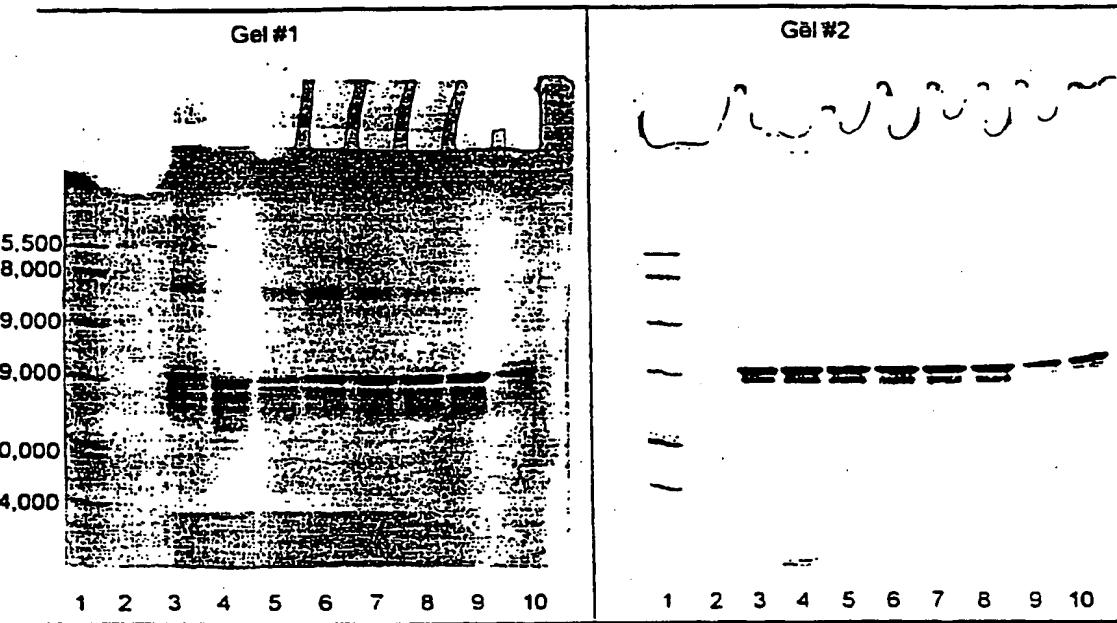


FIGURE 22

27 / 34

Non-reducing SDS-PAGE analysis of Q-Sepharose fractions from Run# I95005



Lane #	Lane Description	Lane #	Lane Description
1	Molecular Weight Markers	1	Molecular Weight Markers
2		2	
3	Load	3	Fraction 5
4	Flow through	4	Fraction 6
5	Fraction 0	5	Fraction 7
6	Fraction 1	6	Fraction 8
7	Fraction 2	7	Fraction 9
8	Fraction 3	8	Fraction 10
9	Fraction 4	9	SC-59735
10	SC-59735	10	SC-59735

FIGURE 23A

28 / 34

Non-reducing SDS-PAGE analysis of Q-Sepharose fractions from run #195005.

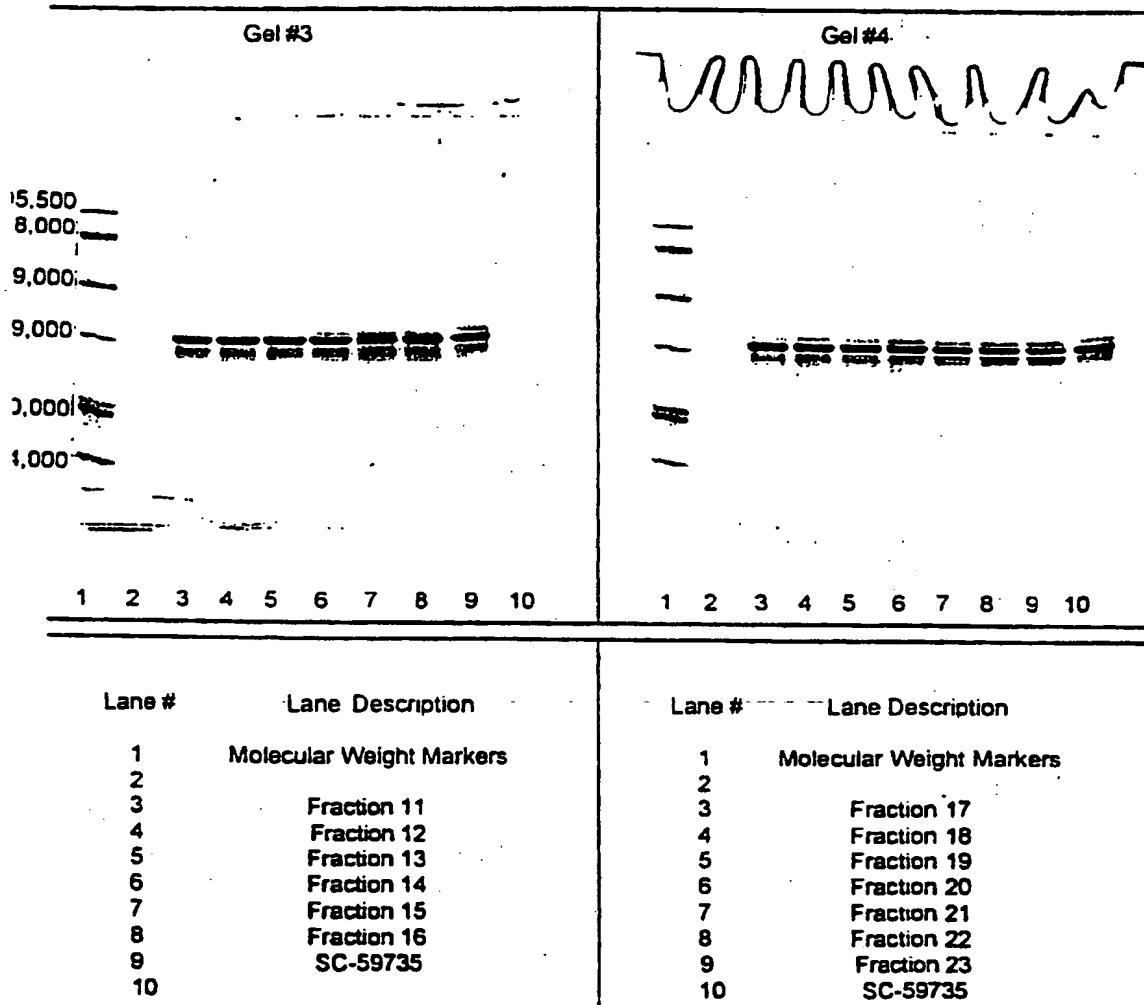
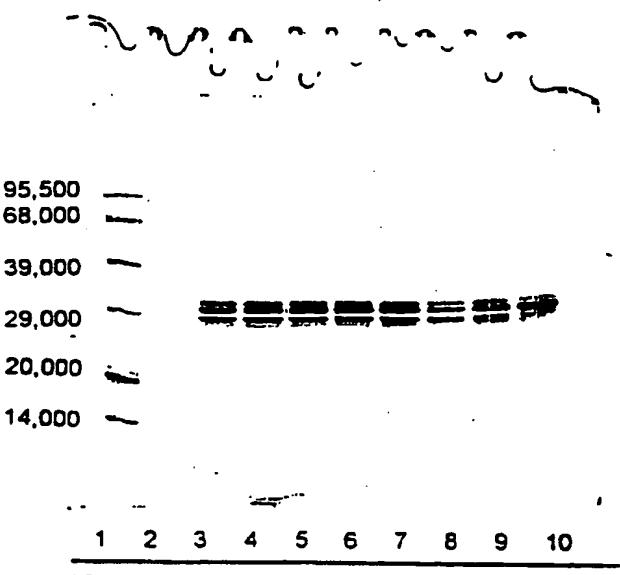


FIGURE 23B

29 / 34

Non-reducing SDS-PAGE analysis of Q-Sepharose fractions from run #195005.

Gel #5



Lane #	Lane Description
1	Molecular Weight Markers
2	
3	Fraction 24
4	Fraction 25
5	Fraction 26
6	Fraction 27
7	Fraction 28
8	Fraction 29
9	Fraction 30
10	SC-59735

FIGURE 23C

30 / 34

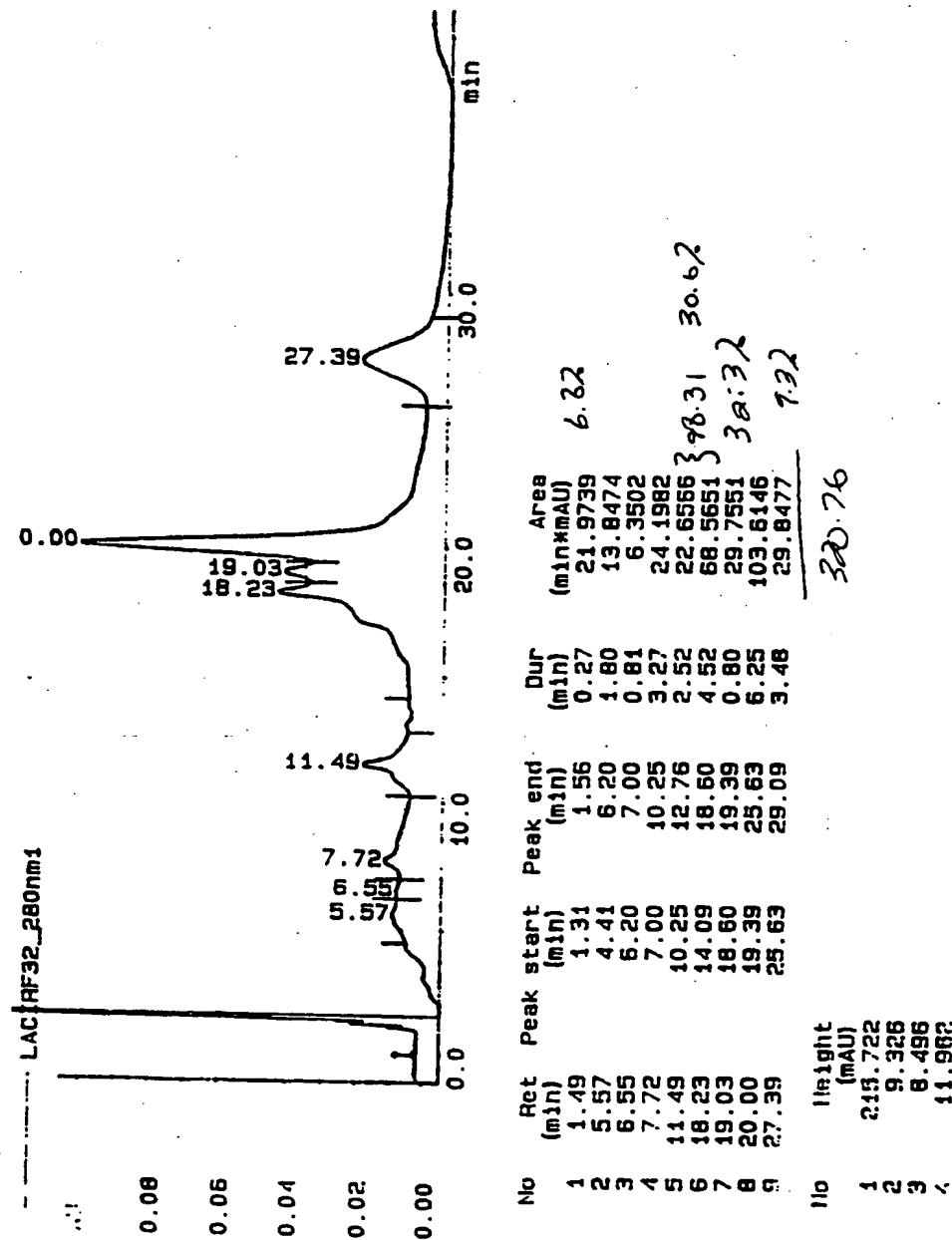
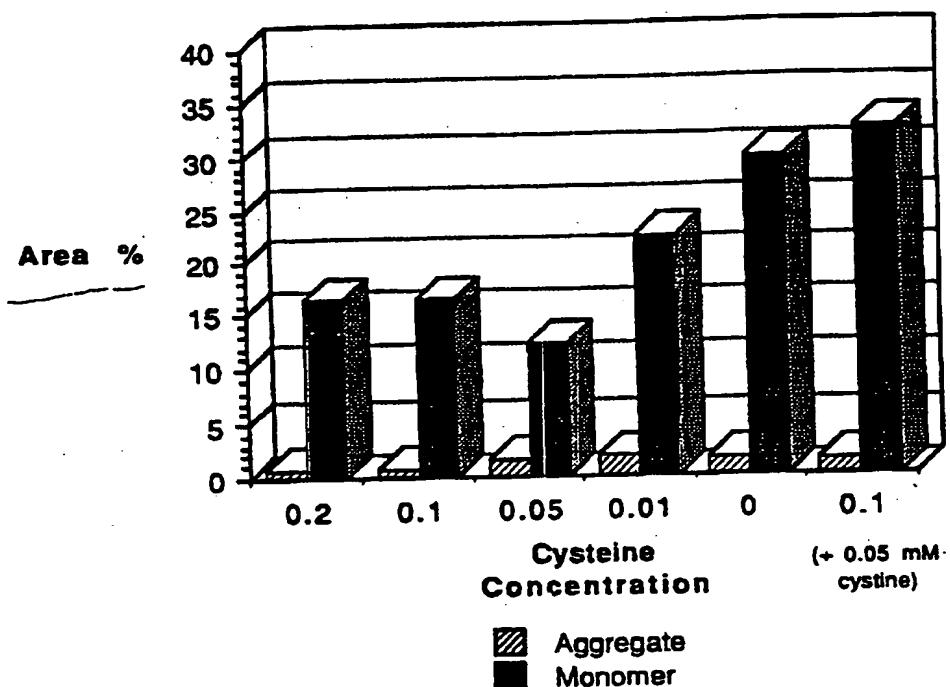


FIGURE 24

31 / 34

Figure 25 Results of SC-59735 refolding in water with 0.4% polyphosphate.**Mono-S Results****FIGURE 25**

32 / 34

Results from experiment evaluating the impact of different polyphosphate chain lengths on SC-59735 refolding.

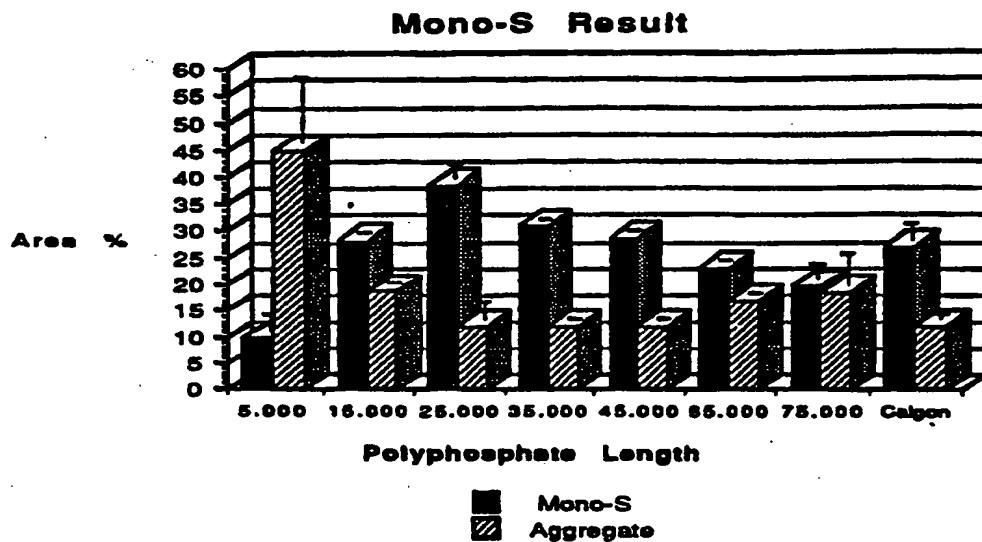


Figure 22 Chain length evaluation expressed as PT.

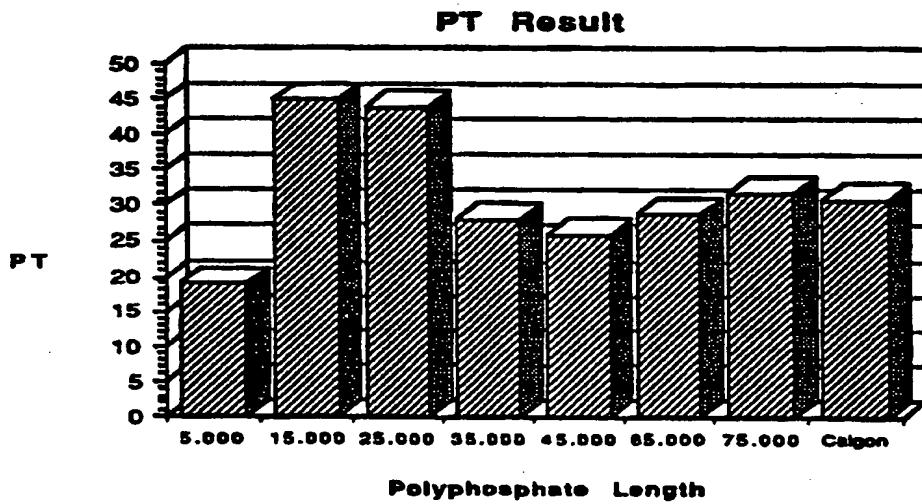
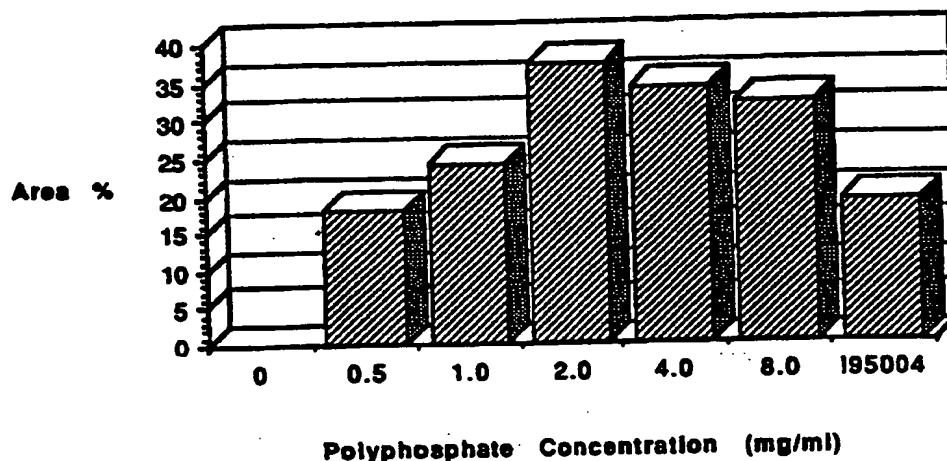


FIGURE 26

33 / 34

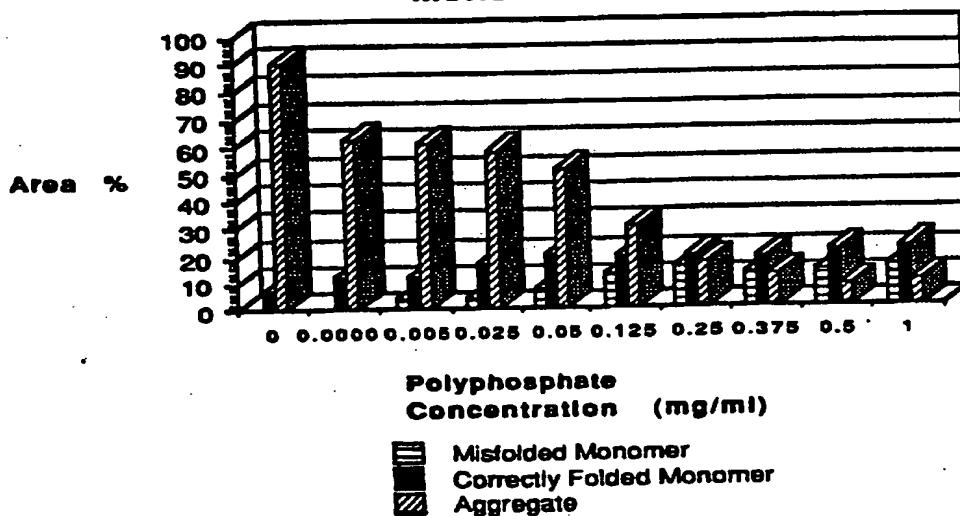
Effects of high concentrations of polyphosphate on SC59735 refold.

**High Polyphosphate Concentration
Mono-S Result**



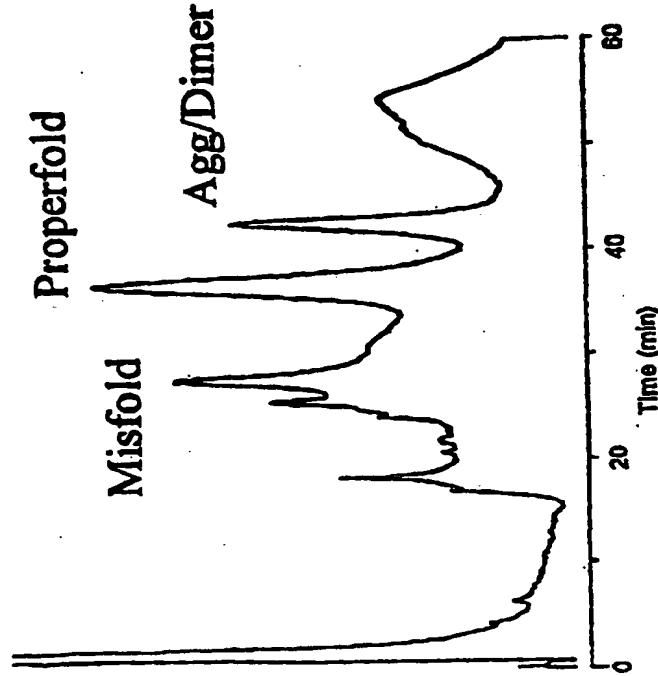
Effects of low polyphosphate concentrations on SC-59735 refolds.

**Low Polyphosphate Concentration
Mono-S Result**

**FIGURE 27**

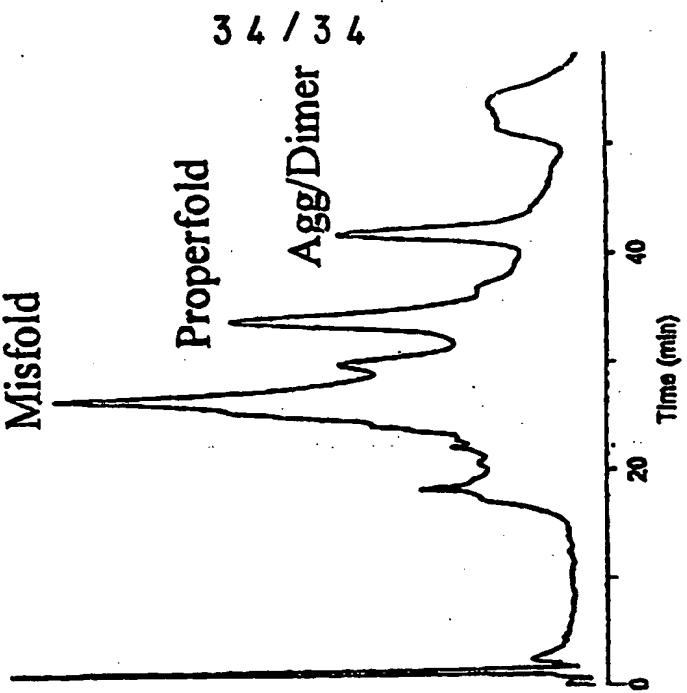
Mono-S Analysis of Refolded TFP

Polyphosphate Refold



Refolding Yields: 30-40%

PEI Refold



15-20%



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Reference P037717EP	Application No./Patent No. 02778529.4 - 2107 PCT/US0232624
Applicant/Proprietor Novartis Vaccines and Diagnostics, Inc.	

COMMUNICATION

The European Patent Office herewith transmits as an enclosure the supplementary European search report under Article 157(2)(a) EPC for the above-mentioned European patent application.

If applicable, copies of the documents cited in the European search report are attached.

Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.

Refund of the search fee

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.





European Patent
Office

**SUPPLEMENTARY
PARTIAL EUROPEAN SEARCH REPORT**

Application Number

which under Rule 45 of the European Patent Convention EP 02 77 8529
shall be considered, for the purposes of subsequent
proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (IPC)									
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim										
X	WO 96/40784 A2 (CHIRON CORP [US]; SEARLE & CO [US]; DORIN GLENN J [US]; ARVE BO H [US]) 19 December 1996 (1996-12-19) * claims 44-70 *	40	INV. A61K38/57									
X	----- US 2001/018204 A1 (PAPATHANASSIU ADONIA E [US] ET AL) 30 August 2001 (2001-08-30) * page 4, paragraph 41 - paragraph 49 * * claims 11-20 *	40										
A	----- BAJAJ M S ET AL: "TISSUE FACTOR PATHWAY INHIBITOR: POTENTIAL THERAPEUTIC APPLICATIONS" THROMBOSIS AND HAEMOSTASIS, STUTTGART, DE, vol. 78, no. 1, 1997, pages 471-477, XP009064749 ISSN: 0340-6245 * page 474, right-hand column, paragraph 2 * ----- -/-	1-39										
			TECHNICAL FIELDS SEARCHED (IPC)									
			A61K									
<p>The supplementary search report has been based on the last set of claims valid and available at the start of the search.</p>												
<p>INCOMPLETE SEARCH</p> <p>The Search Division considers that the present application, or some or all of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for the following claims:</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p>												
<p>1</p> <table border="1"> <tr> <td>Place of search</td> <td>Date of completion of the search</td> <td>Examiner</td> </tr> <tr> <td>Munich</td> <td>23 January 2007</td> <td>Ganschow, Silke</td> </tr> <tr> <td colspan="3"> CATEGORY OF CITED DOCUMENTS <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p> </td> </tr> </table>				Place of search	Date of completion of the search	Examiner	Munich	23 January 2007	Ganschow, Silke	CATEGORY OF CITED DOCUMENTS <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>		
Place of search	Date of completion of the search	Examiner										
Munich	23 January 2007	Ganschow, Silke										
CATEGORY OF CITED DOCUMENTS <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>												



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

EP 02 77 8529

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (IPC)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	TECHNICAL FIELDS SEARCHED (IPC)
A	<p>SPEICH R ET AL: "Efficacy, safety, and tolerance of piperacillin/tazobactam compared to co-amoxiclav plus an aminoglycoside in the treatment of severe pneumonia" EUROPEAN JOURNAL OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES, vol. 17, no. 5, May 1998 (1998-05), pages 313-317, XP002416071 ISSN: 0934-9723 * the whole document *</p> <p>-----</p>	1-39	
1			



European Patent
Office

**INCOMPLETE SEARCH
SHEET C**

Application Number
EP 02 77 8529

Although claims 31-39 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Claim(s) searched completely:
1-30,40

Claim(s) searched incompletely:
31-39

Claim(s) not searched:

Reason for the limitation of the search (non-patentable invention(s)):

Article 52 (4) EPC - Method for treatment of the human or animal body by therapy

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 02 77 8529

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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23-01-2007

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EPO FORM 1055

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Tissue Factor Pathway Inhibitor: Potential Therapeutic Applications

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Introduction

Coagulation at a site of injury is initiated by exposure of blood to cell-surface tissue factor (TF) and formation of the TF/VIIa(VIIa) complex (1,2). The TF/VIIa complex then activates both factors IX and X leading to thrombin generation and fibrin formation (1,2). TFPI (previously known as lipoprotein-associated coagulation inhibitor or extrinsic pathway inhibitor) appears to play a primary role in regulating TF-induced coagulation (1-3). TFPI is primarily synthesized by the endothelium under normal physiologic conditions (4). There are at least three pools of TFPI *in vivo* — ~80-85% remains associated with the endothelial surface presumably bound to cell-surface glycosaminoglycans, ~10% is associated with lipoproteins in plasma and ~3% is present in platelets (1). TFPI has a molecular weight of ~42,000 daltons and consists of three tandem Kunitz-type inhibitory domains (5). The physiologic inhibition of the catalytic activity of TF/VIIa complex occurs in two steps. First, the middle domain of TFPI binds to factor Xa and then its first domain binds to factor VIIa in the TF/VIIa complex (5). The function of third domain is not known with certainty but a segment of it including the C-terminal cationic tail may be involved in binding to the cell-surface glycosaminoglycans (6). The lipoprotein-associated TFPI in plasma is C-terminally truncated and therefore is a less efficient anticoagulant compared to the full-length TFPI (3,7,8). It has also been reported that depletion of TFPI sensitizes rabbits to endotoxin (9) and TF-induced DIC (10), thus establishing an important role for TFPI in the physiology of coagulation. Notably there are no known inherited clinical conditions with TFPI deficiency (11, and Bajaj MS, Bovill E and Bajaj SP, unpublished data).

Abnormal coagulation underlies pathogenesis of many serious illnesses. In particular, induced expression of TF and TF-mediated coagulation occurs in atherosclerotic plaques (12), sepsis (13,14), malignancy (15), ARDS (16), and glomerulonephritis (17). Importantly, in the cell types thought to demonstrate abnormal TF expression in some of the above clinical conditions, TFPI expression is either absent (18) or delayed (19,20). Moreover, plasma levels of

TFPI in above clinical conditions are generally normal or high; however, in some subjects levels have also been found to be low (11). The reason(s) for this is not clear but may be related to the severity of endothelial damage. The elevated plasma TFPI levels may also be due to induced synthesis of TFPI in serum-stimulated fibroblasts and adherent monocytes (19,20) as well as its slightly increased expression by the endothelium during an inflammatory response (21).

Several observations support the need for exogenous TFPI administration to effectively turn off the TF/VIIa complex despite the presence of ~2-fold (~0.2 µg/ml) elevated plasma TFPI levels in several clinical conditions with TF-induced coagulopathy. First and foremost, relatively high concentrations of TFPI are required to inhibit TF/VIIa complex without the need to initially generate factor Xa (22), thus allowing rapid inhibition of TF/VIIa catalyzed coagulation before it is initiated. Use of such high concentrations of TFPI could prove useful in those clinical situations in which a small clot triggered by acute exposure to TF after plaque rupture or angioplasty could cause serious consequences. Second, the plasma TFPI levels achieved during a severe coagulopathy such as sepsis are in the range of 0.2 µg/ml whereas efficacy studies indicate a need for therapeutic levels of full length rTFPI in the range of 1–2 µg/ml. (11). Third, plasma TFPI levels do not reflect the amount of TFPI associated with the endothelial cell surface and maybe insufficient to control ongoing intravascular coagulation. And, fourth, the TFPI released due to endothelial damage may be C-terminally truncated, and therefore a less efficient anticoagulant (3,7,8). The above considerations therefore justify administration of exogenous TFPI despite its elevated plasma levels in several clinical conditions where coagulation plays a role in the pathogenesis of disease. Preclinical studies discussed in this article suggest that this may indeed be the case.

Two forms of rTFPI are available for therapeutic purposes—full-length rTFPI and the two-domain rTFPI in which the third Kunitz domain and the C-terminal region has been deleted. These two forms of rTFPI have different pharmacokinetics and activity profiles. First, full-length rTFPI binds to factor Xa at a much faster rate than the two-domain rTFPI; as discussed earlier, this results in faster inhibition of TF/VIIa complex (3,8) and is potentially more likely to be useful when rapid inhibition of TF-induced coagulation is required. Second, in contrast to the two-domain rTFPI, full-length rTFPI binds to endothelia

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glycosaminoglycans in microvessels (6) and therefore might be preferable for protection against microvascular thrombosis. Third, the two forms of rTFPI also differ in their clearance from plasma after intravenous administration. Because of its binding to the cell surface glycosaminoglycans, full-length rTFPI is cleared rapidly from circulation and therefore it may be difficult to target it to a focal area of arterial wall injury unless administered locally via an indwelling catheter. In contrast, the two-domain rTFPI persists in circulation for a longer time (23) and may be potentially more useful in situations where a generalized anticoagulant effect is desired, such as in sepsis or when TF-containing vesicles from tumor cells are continuously entering the circulation as in Troussseau syndrome. Unless specified by the authors of the multiple studies reviewed in this paper, rTFPI was assumed to mean full-length rTFPI. Also, in this article, unless specifically stated, rTFPI means full-length rTFPI.

Sites of TFPI Synthesis

Under normal physiologic conditions, TFPI is synthesized primarily by the microvascular endothelium (Fig. 1) and in small amounts by megakaryocytes and macrophages (4,18). It is not synthesized by normal hepatocytes or by the endothelium of large vessels (4,18). Human fibroblasts express negligible amounts of TFPI; however, upon serum-stimulation the TFPI expression by these cells is increased by 6-8 fold (Fig. 2) (19). Similarly circulating monocytes express none to very little TFPI; however, monocytes adherent to biologic ligands express significant amounts of TFPI (Fig. 3) (20). As is the case with endothelial cells, TFPI expressed by the stimulated fibroblasts and adherent monocytes stays associated with the cell surface. Thus, these three cell types appear to regulate intravascular as well as extravascular clotting.

Potential Therapeutic Applications

Coronary Artery Disease, Arterial Thrombosis and Restenosis

Disruption of atherosclerotic plaque complicated by acute thrombosis is known to underlie acute coronary syndromes (24). Several studies have provided evidence of ongoing coronary thrombosis in patients with unstable angina and myocardial infarction (25,26). In these patients a hypercoagulable state has also been observed (27). Moreover, two studies have reported the presence of TF in macrophages and monocytes in atherosclerotic plaques (12,28). It is likely that the rupture of an atheromatous plaque exposes this TF to circulating blood with local thrombus formation. Indeed, the most thrombogenic site in the plaque is the lipid-rich core (29) where TF is present in abundance (24). The mural thrombus formed on a ruptured plaque is resistant to conventional anticoagulation and continues to grow despite administration of high doses of heparin as well as aspirin (30).

It appears that inhibition of TF may be a reasonable

approach to preventing acute coronary and vascular thrombosis. Three animal studies have addressed the possibility of inhibiting vascular thrombosis by the use of anti-TF antibodies. In the first study, Jang et al. (31) exposed the adventitial TF to circulating blood in an eversion (inside out) graft model of rabbit femoral artery. A 2 hr infusion of a neutralizing anti-TF monoclonal antibody prevented thrombosis in 80% of the animals. In the second study, Pawashe et al. (32) reported inhibition of vascular thrombosis in a stenotic injured rabbit carotid artery model by an anti-TF monoclonal antibody. In the third study, Speidel et al. (33) also observed inhibition of coagulation on the intimal surface of the balloon injured rabbit aorta by an anti-TF antibody. Thus, in all three studies an anti-TF antibody was efficacious in inhibiting thrombosis.

A few studies have also investigated the role of TFPI in inhibiting TF-induced vascular thrombosis. TFPI purified from plasma has been found *in vitro* to inhibit fibrin formation on the procoagulant subendothelial matrix in a flow model (34). In another study, Sobel and coworkers (35) induced intimal injury and thrombosis in the dog femoral artery using electrical current followed by recanalization with tissue plasminogen activator (t-PA). Following the infusion of t-PA, none of the dogs infused with rTFPI developed reocclusion whereas reocclusion occurred in all of the control animals. In a subsequent study by the same group (36) in which intimal injury was induced in the dog coronary artery, it was also found that rTFPI maintains coronary patency following fibrinolysis. The results of these two studies are encouraging since aspirin and heparin, currently used following thrombolysis, are only partially useful in preventing recurrent thrombosis (37).

Restenosis following arterial interventions is caused by intimal hyperplasia, which in turn has been suspected to result from thrombus formation following arterial injury. In two studies, the effect of rTFPI on restenosis following intimal injury in animal models was examined and it was found that rTFPI causes a significant reduction in the incidence of restenosis (38,39). Thus, it appears that rTFPI may be beneficial in multiple cardiovascular conditions with underlying TF-induced coagulation such as unstable angina, following fibrinolysis and percutaneous transluminal coronary angioplasty (PTCA), as well as to prevent restenosis following arterial interventions.

Microvascular Anastomosis

TF expressed in the subendothelial layer of blood vessels and exposed to the circulating blood is an important cause of thrombosis of microvascular anastomosis particularly in patients with extremity trauma. Khouri et al. (40) administered topical rTFPI to a rabbit ear artery after microvascular repair. The patency rates post-operatively at day 7 were as follows: untreated control -0%, heparin irrigation (10 U/ml) -40% and rTFPI (20 µg/ml) -73% ($P < 0.0005$). Another study used a rabbit ear model of microvascular surgery with irrigation of vascular lumen with rTFPI (41). Ear survival was 95% for animals



Fig. 1 Immunoperoxidase staining TFPI antigen expressed by human lung microvascular endothelial cells grown in culture. The primary antibody used was rabbit anti-human TFPI IgG and the secondary antibody was biotinylated sheep anti-rabbit IgG. The reaction was developed with diaminobenzamidine. Rabbit preimmune IgG was used in the negative control experiment. A (left), negative control; B (right), positive experiment.

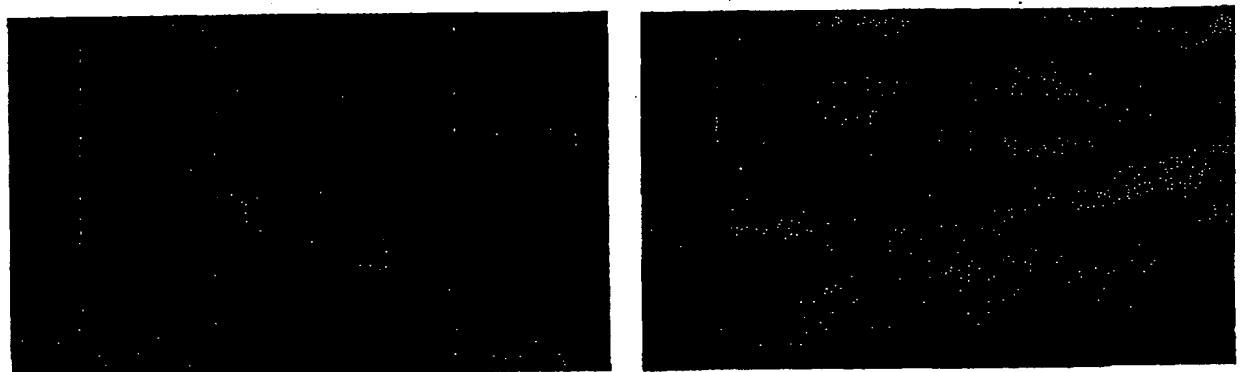


Fig. 2 Immunofluorescence localization of TFPI antigen expressed by serum-stimulated human fetal lung fibroblasts. The primary antibody used was rabbit anti-human TFPI IgG and the secondary antibody was FITC conjugated goat anti-rabbit IgG. Rabbit preimmune IgG was used in the negative control experiments. A (left), negative control; B (right), positive experiment.

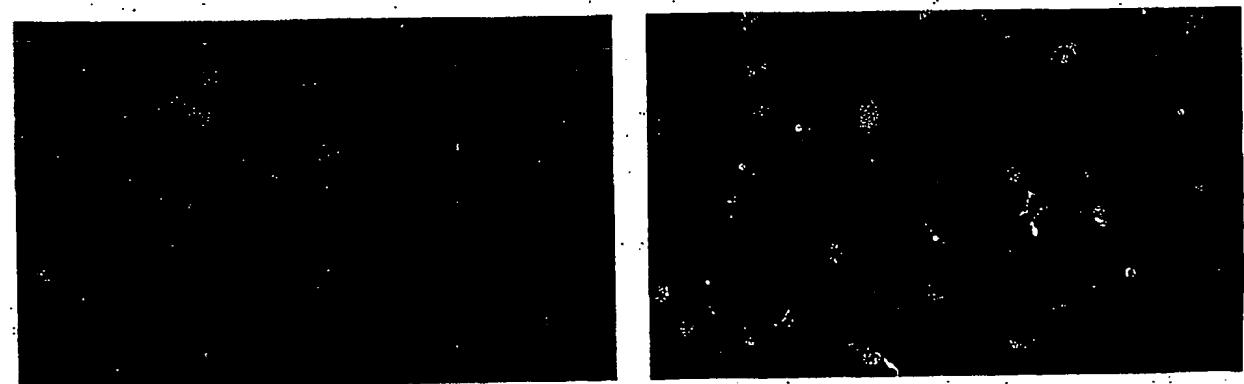


Fig. 3 Immunofluorescence demonstrating expression of TFPI by human peripheral blood monocytes adherent to fibronectin. Primary and secondary antibodies are as in Fig. 2. A (left), negative control; B (right), positive experiment.

receiving rTFPI (4–40 µg/ml) compared to 20% in the untreated controls. Further, in a whole vessel model of microvascular anastomosis, rTFPI reduced generation of thrombin on the vascular endothelium (42). Based upon these studies the clinical efficacy of rTFPI in microvascular surgery is currently being evaluated.

Stroke and Ischemia-Reperfusion (I/R) Injury

Activation of coagulation due to I/R injury has been implicated in the microvascular perfusion defects in a baboon model of experimental acute thrombotic stroke (43). Further, in a baboon model of reversible middle cerebral artery occlusion, an anti-TF antibody prevented TF-mediated coagulation and microvascular perfusion defects (44). In another study, Ragni et al. (45) found that an anti-TF monoclonal antibody inhibited vascular reocclusion after thrombolysis in a rabbit model of carotid artery thrombosis. Since TFPI also inhibits TF-induced coagulation, it may have potential therapeutic applications in stroke and in I/R injury to the brain and possibly other organs such as lung, liver, kidney and the spinal cord. In this regard, one study examined the protective effect of rTFPI against I/R injury in spinal cords of rabbits. Eighty-eight percent of the animals treated with rTFPI versus 20% treated with heparin recovered neurologic function (46). In another study, Kraemer et al. (47) demonstrated a beneficial effect of rTFPI on tissue survival in a rabbit ear model of I/R injury. Thus, rTFPI could potentially be used in the setting of I/R injury following surgery, shock or acute thrombosis.

Sepsis with DIC

Sepsis is a common cause of morbidity and mortality in the hospitalized patient. Endotoxin and cytokines released during sepsis activate monocytes and endothelium to express TF (13,48). In contrast, cultured endothelial cells stimulated with cytokines or endotoxin upregulate TFPI expression only very slightly (21). Thus, the anticoagulant surface of the vascular endothelium converts to a procoagulant phenotype when perturbed by various inflammatory mediators of sepsis; this leads to DIC and thrombosis with or without consumption (48). Although TFPI levels in plasma from patients with sepsis and DIC are generally elevated possibly due to endothelial damage (1), administration of exogenous rTFPI and anti-TF agents could potentially be useful for several reasons mentioned above. In a study by Wildgoose et al., endotoxin-induced DIC in rabbits was inhibited by administration of inactivated factor VIIa (49). Moreover, Bregengard et al. (50) demonstrated that two-domain rTFPI inhibited endotoxin-induced DIC in rabbits. Further, in a baboon model of DIC with *E. coli* septic shock, administration of an anti-TF antibody inhibited DIC had anti-inflammatory effects and prevented mortality (51). In the above baboon model of *E. coli* sepsis, administration of rTFPI decreased IL-6 levels (52) and was also found to decrease mortality (52,53). In another study, endotoxin-induced coagulation in chimpanzees was inhibited by an anti-TF antibody (54) and monoclonal Fab fragments

against factor VIIa (55). Further, Johnson et al. (56) found that rTFPI inhibited IL-8 synthesis, which was induced synergistically by coagulation and endotoxin in a human whole blood culture system. Thus, cumulatively, the efficacy of rTFPI in inhibiting both the intravascular coagulation and the inflammatory response in animal models of sepsis with DIC appears to be convincing. A phase 2 clinical trial of rTFPI in sepsis is presently in progress.

ARDS

ARDS is a severe pulmonary dysfunction complicating sepsis, trauma, aspiration, etc. It is characterized by both intravascular (57) and extravascular (58) coagulation and fibrin deposition. Idell et al. (58) have demonstrated an increased activity of the extrinsic coagulation pathway in the alveolar compartment of the lungs in patients with ARDS. Furthermore, endothelium is severely perturbed in this syndrome. In this context, we have demonstrated a significant increase in plasma levels of three endothelial-specific proteins, namely, TFPI (59), von Willebrand factor antigen (vWF-Ag) (59), and thrombomodulin (unpublished observations) in patients with ARDS. In general, plasma levels of these three proteins were elevated to a lesser degree in patients at-risk for ARDS. To date, no animal studies investigating the efficacy of anti-TF agents have been reported in ARDS. Nonetheless, one might speculate that inhibiting TF-induced coagulation in the lung despite the elevated plasma TFPI levels might be one mode of therapy that could be offered in this highly complex multifactorial disease.

Deep Venous Thrombosis (DVT)

DVT is a frequent complication in the hospitalized patient. Three studies have reported the usefulness of rTFPI in two different animal models of venous thrombosis. In the first study, Spokas et al. (60) investigated the efficacy of rTFPI at 400–800 µg/kg, given as IV infusion in a rabbit model with vena caval thrombosis induced by a crush injury combined with stasis. rTFPI was significantly effective in preventing/decreasing the size of the thrombus in 83% of the animals. However, in this study, efficacy of rTFPI was not compared to any other known antithrombotic agent. In the two other studies, Holst et al. (61,62) compared the efficacy of two-domain rTFPI to that of low molecular weight heparin in venous thrombosis induced in rabbit jugular veins. Two-domain rTFPI (1.0–10.0 mg/kg) was found to be as effective as low molecular weight heparin; further it had no hemorrhagic side effects. Of note, injury followed by exposure of TF in the venous wall with TF-induced coagulation, is unusual in the pathophysiology of DVT, which is felt to be primarily due to stasis. Thus it is doubtful that rTFPI will be routinely used as the first line of therapy in DVT, although it is likely that it may serve as an alternative to heparin and low molecular weight heparin under those circumstances where complications of heparin therapy are noted.

Malignancy

Thromboembolic complications are an important cause of morbidity and mortality in patients with cancer. Several solid malignant tumors such as tumors of breast, lung, pancreas, colon and stomach, stain positively for TF (63). Membrane fragments containing TF are shed into the circulation by tumor cells and may contribute significantly to the hypercoagulable state in cancer (15,64). Kakkar et al. (65) recently reported activation of the extrinsic pathway of coagulation in patients with solid tumors. Interestingly, the plasma TFPI levels are also increased in patients with certain types of cancers (66). However, as noted above, a hypercoagulable state exists in these patients despite the presence of elevated plasma TFPI levels and it is conceivable that rTFPI could be used to inhibit ongoing activation of coagulation.

Notably, a new role for TF in the metastasis of malignant tumors has recently become evident. Endothelial TF expression in human breast tumors correlated with malignant phenotype and was found to be a marker of angiogenesis (67). Further, Meth A sarcoma cell lines overexpressing TF were found to have higher mitogenic and angiogenic activity (68), and melanoma cell lines expressing TF were less invasive after treatment with anti-TF antibody (69). These authors (69) also demonstrated that the cytoplasmic domain of TF and not its procoagulant extracellular domain was necessary for metastasis. Whether or not the use of anti-TF antibodies or rTFPI in animal models of tumors will impede tumor growth and metastasis remains to be determined.

Conclusion

Anti-TF antibodies and rTFPI have been found to be effective in animal models of several diseases in which TF-induced coagulation plays a major role. The results of these studies are summarized in Table 1. In studies performed in primates, the plasma rTFPI levels needed for therapeutic efficacy were ~1–2 µg/ml and could be monitored by PI assays (11). The therapeutic dose of rTFPI was determined to be ~20 mg/kg/day (11). Importantly, no bleeding complications were reported at this dose and ~10-fold higher concentrations were required for bleeding complications to occur. Thus, it appears that rTFPI is a safe drug when administered at these therapeutic doses. Two-domain rTFPI appears to be a much less potent anticoagulant as compared to full-length rTFPI and thus may be less effective in clinical situations where a rapid antithrombotic effect is required. Two-domain nonglycosylated rTFPI has a significantly longer half-life (23) and a prolonged antithrombotic effect, which may be of advantage in certain clinical situations. Full-length rTFPI is presently being used in clinical trials in patients with sepsis and DIC and in those following microvascular surgery. It could also be used in other diseases complicated by TF-induced coagulation such as unstable angina and to prevent thrombosis following fibrinolysis or PTCA for coronary artery disease. rTFPI also has the potential to be used in patients with early stroke or transient ischemic attacks as well as to minimize ischemia-reperfusion injury. Lastly, it may have a therapeutic value in those conditions which are

Table 1 Potential therapeutic indications for TFPI; status of preclinical and clinical trials

Clinical condition	Animal model	Anti-TF agent used	rTFPI clinical trial
Coronary artery disease, arterial thrombosis, restenosis	(a) Rabbit femoral artery thrombosis (30)	Anti-TF antibody	None
	(b) Stenotic injured rabbit carotid artery model (31)	Anti-TF antibody	
	(c) Dog femoral artery injured by electrical current; thrombolysis with t-PA (34)	rTFPI infusion	
	(d) Dog coronary arteries injured by electrical current (35)	rTFPI infusion	
	(e) Restenosis (intimal injury) (37,38)	rTFPI infusion	
Microvascular anastomosis	(a) Microvascular repair of crushed rabbit ear artery (39)	rTFPI-intra-arterial irrigation	Trial in progress
	(b) Microvascular surgery rabbit ear artery (40)	rTFPI-intra-arterial irrigation	
Stroke and ischemia reperfusion injury	(a) Middle cerebral artery occlusion in baboon (43)	Anti-TF antibody	None
	(b) Rabbit carotid artery (44)	Anti-TF antibody	
	(c) I/R injury-rabbit spinal cord (45) -rabbit ear model (46)	rTFPI infusion	
Sepsis with DIC	(a) Rabbit, endotoxin-induced DIC (48,49)	Inactivated FVIIa; 2 domain rTFPI	Phase II trials in progress
	(b) Baboon-E. coli sepsis with DIC (50)	Anti-TF antibody	
	(c) Baboon-E. coli sepsis with DIC (51,52)	rTFPI infusion	
	(d) Chimpanzee-endotoxin-induced DIC (53)	Anti-TF antibody	
	(e) Chimpanzee-endotoxin-induced DIC (54)	Factor VII/VIIa Fab fragment	
Deep venous thrombosis	(a) Rabbit vena caval thrombosis (60)	rTFPI	None
	(b) Rabbit interval jugular vein thrombosis (61,62)	Truncated rTFPI	

accompanied by intravascular coagulation complicated by thrombocytopenia and risk for bleeding complications from conventional anticoagulant therapy.

Summary

Tissue factor pathway of coagulation plays a dominant role during normal haemostasis. Tissue factor pathway inhibitor (TFPI), expressed primarily by the microvascular endothelium, appears to be the major physiologic inhibitor of TF-induced coagulation. TF-initiated coagulation also plays an important role in the pathophysiology of many diseases including coronary thrombosis, sepsis, disseminated intravascular coagulation, stroke, cancer, acute respiratory distress syndrome, and ischemia-reperfusion injury. Several animal studies have found a beneficial effect of anti-TF monoclonal antibodies and recombinant TFPI in some of the above clinical conditions. rTFPI is presently being used in clinical trials in patients with sepsis and in those following microvascular surgery. This article discusses many of the animal studies addressing inhibition of TF-induced coagulation, as well as potential therapeutic uses of rTFPI in humans.

Acknowledgement

The authors thank Dr. S.I. Rapaport for his critical review of the manuscript and many useful suggestions.

References

accompañado por la trombocitopenia e riesgo de complicaciones de sangrado de la terapia anticoagulante convencional.

Summary

Tissue factor pathway of coagulation plays a dominant role during normal haemostasis. Tissue factor pathway inhibitor (TFPI), expressed primarily by the microvascular endothelium, appears to be the major physiologic inhibitor of TF-induced coagulation. TF-initiated coagulation also plays an important role in the pathophysiology of many diseases including coronary thrombosis, sepsis, disseminated intravascular coagulation, stroke, cancer, acute respiratory distress syndrome, and ischemia-reperfusion injury. Several animal studies have found a beneficial effect of anti-TF monoclonal antibodies and recombinant TFPI in some of the above clinical conditions. rTFPI is presently being used in clinical trials in patients with sepsis and in those following microvascular surgery. This article discusses many of the animal studies addressing inhibition of TF-induced coagulation, as well as potential therapeutic uses of rTFPI in humans.

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Article

Efficacy, Safety, and Tolerance of Piperacillin/Tazobactam Compared to Co-Amoxiclav plus an Aminoglycoside in the Treatment of Severe Pneumonia

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Abstract An open, randomized, multicenter study was conducted to compare the efficacy and safety of piperacillin/tazobactam and co-amoxiclav plus aminoglycoside in the treatment of hospitalized patients with severe community-acquired or nosocomial pneumonia. Of the 89 patients who entered the study, 84 (94%) were clinically evaluable. A favorable clinical response was observed in 90% of the piperacillin/tazobactam group and in 84% of the co-amoxiclav/aminoglycoside group (not significant). The bacteriological efficacy was comparable in both groups (96% vs. 92%; not significant). There was only one fatal outcome in the piperacillin/tazobactam group compared to six in the co-amoxiclav/aminoglycoside group regimen ($P=0.058$). The adverse event rate was non-significantly lower in the piperacillin/tazobactam group compared to the co-amoxiclav/aminoglycoside group (2% vs. 7%; $P=0.32$). Piperacillin/tazobactam is safe and highly efficacious in the treatment of serious pneumonia in hospitalized patients. It compares favorably with the combination of co-amoxiclav/aminoglycoside.

Introduction

Piperacillin is a semi-synthetic penicillin with a broad spectrum of antibacterial activity. Administered parenterally, it has been widely used in the treatment of serious infections, including pneumonia [1–3]. However, the growing prevalence of β -lactamase-producing or-

ganisms increasingly limits the clinical efficacy of piperacillin as monotherapy [4]. Tazobactam, a recently developed penicillanic acid sulfone, irreversibly inhibits a wide range of bacterial β -lactamases. Consequently, tazobactam prevents the inactivation of piperacillin by β -lactamase-producing microorganisms such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Escherichia coli*, *Klebsiella pneumoniae*, and anaerobes [5–7].

In two recent trials, the combination of piperacillin and tazobactam was effective, well tolerated, and safe in the treatment of hospitalized patients with pneumonia [8, 9]. Furthermore, two randomized, comparative, multicenter trials indicate that piperacillin/tazobactam is as effective as cefuroxime and more effective than ceftazidime in this same indication (A.P. Pallett and M.P. Carroll, 6th International Congress for Infectious Diseases, Prague, 1994, Abstract no. 857; M. Joshi et al., 6th International Congress for Infectious Diseases, Prague, 1994, Abstract no. 856). The present study was designed to compare the efficacy, tolerance, and safety of piperacillin/tazobactam with that of co-amoxiclav plus aminoglycoside in patients with serious community-acquired or nosocomial pneumonia.

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Materials and methods

Entry Criteria. Participants included patients of either sex ≥ 16 years of age with pneumonia defined by the presence of three criteria: i) body temperature $>38^\circ\text{C}$; ii) new or progressive infiltrates on chest radiographs or focal signs on physical examination of the chest, and iii) at least two of the following: new onset of purulent sputum (≥ 25 leukocytes and ≤ 10 epithelial cells per low power microscopical field) or signs of infection (elevated C-reactive protein, elevated erythrocyte sedimentation rate, leukocytosis); respiratory pathogen isolated from blood culture; isolation of pathogen from sputum or specimen obtained by bronchoalveolar lavage, bronchial brushing or biopsy. The severity of the changes on the initial chest radiographs was scored as follows: 1, mild: unilobar infiltrates; 2, moderate: bilobular infiltrates; 3, severe: bilateral infiltrates. Only patients with at least one of the following criteria indicating nosocomial or serious infections were included: i) hospital-acquired infection (at least 48 h after admission); ii) underlying disease (diabetes, alcoholism, chronic bronchitis, collagen vascular disease); iii) the presence of at least two of the following clinical signs: diastolic blood pressure ≤ 60 mmHg; respiratory rate ≥ 30 /min; $\text{PaO}_2 \leq 6.6$ kPa.

Exclusion criteria included known allergy to any of the study drugs; history of cystic fibrosis, tuberculosis, or respiratory tract carcinoma; antibiotic therapy within 12 h of enrollment; HIV infection; neutropenia ($<1000/\text{mm}^3$) or thrombopenia ($<50000/\text{mm}^3$); septic shock; hemodialysis, peritoneal dialysis, plasmapheresis or hemoperfusion; elevated serum transaminases, alkaline phosphatase or bilirubin (>3 above the normal values); previous treatment with piperacillin/tazobactam or co-amoxiclav/aminoglycoside during the present hospital stay; pregnancy or breast feeding.

Study Design. This open, randomized, comparative study was conducted in five hospital centers in Switzerland. Patients were randomized to receive either piperacillin/tazobactam or co-amoxiclav/aminoglycoside in a 1:1 ratio according to a computer-derived program. Both regimens were administered as 30-min i.v. infusions. The piperacillin/tazobactam treatment consisted of a combination of 4 g of piperacillin and 500 mg of tazobactam at 8 h intervals, while the co-amoxiclav/aminoglycoside group received a combination of 2 g of amoxicillin and 200 mg of clavulanic acid at 8 h intervals, plus a single dose of 3–6 mg/kg of aminoglycoside (netilmicin or gentamicin). The patients were to be treated for a minimum of 48 h and a maximum of 21 days.

Outcome Measures. During the period of antibiotic treatment, routine clinical evaluations were performed daily during the first week, and every 2 to 3 days thereafter. Laboratory parameters were determined on day 4 and on the last day of treatment. Bacteriological cultures were repeated on day 4 and, if possible, at the cessation of the study, and all concomitant therapy was recorded in detail. Ten to 14 days following the cessation of treatment, the patient's general status was assessed.

The clinical response to therapy was classified as follows: non-assessable, <48 h of therapy; cure, clinical response with disappearance of fever, tachypnea, and other clinical signs (except for chest x-ray) and symptoms during the treatment period; improvement, marked reduction of the signs and symptoms of infection, without complete resolution; relapse, adequate initial response followed by a worsening of the clinical condition due to the occurrence of a bacterial or fungal infection within 7 days of stopping treatment; failure, no response to at least 48 h of antibiotic therapy, worsening of the clinical condition due to infection, or death.

The bacteriological efficacy was assessed in patients treated for at least 48 h and exhibiting one or more baseline pathogens according to the following classification: documented eradication, all baseline pathogens eradicated and no new pathogens present in fol-

low-up cultures; presumed eradication, no repeat sputum sample could be obtained in a patient with a favorable clinical response; documented persistence, any baseline pathogen was present in a culture or cultures obtained from any site of infection upon completion of therapy; presumed persistence, unfavorable clinical outcome, but no follow-up cultures were available; super-infection, all baseline pathogens were eradicated, but one or more new pathogens appeared in the follow-up cultures; indeterminate, switch of antibiotic therapy, concomitant antibiotic therapy for reasons other than failure, death during therapy for a reason not related to the infection, or <48 h of therapy.

All adverse events occurring during the trial were recorded. The possible relationship of an adverse event to treatment was assessed as definite, probable, possible, remote, not related, or unknown. The safety analysis was performed on all patients who were enrolled in the study (intent-to-treat).

Statistical Methods. All values are presented as the mean \pm SD. Frequencies and categories were compared using Fisher's exact test. The Mann-Whitney U test was used for continuous variables. A P value <0.05 was considered significant.

Results

Patient Characteristics. A total of 89 patients were enrolled: 44 in the piperacillin/tazobactam group and 45 in the co-amoxiclav/aminoglycoside group. The two groups were highly comparable regarding demographic characteristics, clinical signs, and laboratory as well as radiographic parameters of severity of pneumonia (Table 1). Ten patients had nosocomial pneumonia and 79 serious community-acquired pneumonia.

Clinical Efficacy. For the clinical efficacy evaluation, five patients had to be excluded (3 in the piperacillin/tazobactam group and 2 in the co-amoxiclav/aminoglycoside group) for the following reasons: treatment duration less than 48 h ($n=4$), and erroneous inclusion of a patient with urinary tract infection ($n=1$). The mean duration of antibiotic therapy in all patients was 10.2 days for the piperacillin/tazobactam group and 10.1 days for the co-amoxiclav/aminoglycoside group. In the clinically evaluable patients, the mean duration of treatment was 10.7 days for the piperacillin/tazobactam group and 10.5 days for the co-amoxiclav/aminoglycoside group.

The results of the clinical efficacy evaluation (84 patients) are presented in Table 2. Clinical cure was achieved in 33 (81%) of the patients treated with piperacillin/tazobactam and in 28 (65%) of the patients who received co-amoxiclav/aminoglycoside ($P=0.091$). An overall favorable clinical response (cure or improvement) was observed in 37 (90%) of the piperacillin/tazobactam patients and in 36 (84%) of the co-amoxiclav/aminoglycoside patients ($P=0.288$). There were three (7%) treatment failures in the piperacillin/tazobactam group and six (14%) in the co-amoxiclav/aminoglycoside group ($P=0.266$). One patient in each group relapsed.

Table 1 Demographic characteristics and clinical data at presentation of patients treated with either piperacillin/tazobactam or co-amoxiclav/aminoglycoside

Characteristic	PIP/TAZ group (n = 44)	Co-AMX/amino group (n = 45)	P value
Age (years)*	64.7 ± 18.7 4/4	64.6 ± 17.0 6/45	>0.5 0.38
Nosocomial pneumonia	38.9 ± 0.8	38.6 ± 0.9	0.11
Leukocytes (10 ⁹ /l)*	13.7 ± 6.1	15.0 ± 6.6	0.48
C-reactive protein (mg/l)*	185 ± 104	186 ± 125	>0.5
Serum urea (mmol/l)*	12.2 ± 25	17.1 ± 39.1	0.47
Albumin (g/l)	35 ± 7	34 ± 8	>0.5
Radiographic score*	2.3 ± 0.8	2.3 ± 0.7	>0.5

* Values expressed as mean ± SD.

PIP, piperacillin; TAZ, tazobactam; Co-AMX, co-amoxiclav; amino, aminoglycoside

Table 2 Clinical efficacy in evaluable patients treated with either piperacillin/tazobactam or co-amoxiclav/aminoglycoside

Outcome	No. (%) of patients		P value
	PIP/TAZ group (n = 41)	Co-AMX/amino group (n = 43)	
Cure*	33 (81)	28 (65)	0.09
Improvement ^b	4 (10)	8 (19)	0.2
Relapse ^c	1 (2)	1 (2)	>0.5
Failure ^d	3 (7)	6 (12)	0.26

* Clinical response with disappearance of fever, tachypnoea, and other clinical signs (except for chest x-ray) and symptoms during the treatment period.

^b Marked reduction of the signs and symptoms of infection, without complete resolution.

^c Adequate initial response followed by a worsening of the clinical condition due to the occurrence of a bacterial or fungal infection within 7 days of stopping treatment.

^d No response at least 48 h of antibiotic therapy, worsening of the clinical condition due to infection, or death.

PIP, piperacillin; TAZ, tazobactam; Co-AMX, co-amoxiclav; amino, aminoglycoside.

Whereas there was one death in the piperacillin/tazobactam group, six deaths occurred among patients receiving co-amoxiclav/aminoglycoside ($P=0.0586$; $P=0.0584$ in the intent-to-treat analysis, which included all 89 patients). All causes of death were attributable to pneumonia, either due to respiratory failure, irreversible septic shock, or multiple organ failure. None of these deaths were attributed to the study medication.

Bacteriological Efficacy. For the analysis of bacteriological efficacy, it was necessary to exclude 19 patients in the piperacillin/tazobactam group and 18 patients in the co-amoxiclav/aminoglycoside group. Bacteriological non-evalutability was due to the absence of a baseline pathogen ($n=32$), treatment duration of less than 48 h ($n=4$), and incorrect diagnosis ($n=1$). Bacteria were isolated in 25 of the 41 clinically evaluable patients (61%) in the piperacillin/tazobactam group and in 27 of the 43 evaluable patients (63%) in the co-amoxiclav/aminoglycoside group. The majority of the patients were infected with a single pathogen; four patients had two pathogens, and one patient had three. *Streptococcus pneumoniae* was the most common pathogen. It was isolated in 14 of the 25 (56%) piperacillin/tazobactam patients, and 13 of the 27 (48%) co-amoxiclav/aminoglycoside patients. The second most common pathogen was *Haemophilus influenzae*, which

was detected in four patients in each group. Twenty other pathogens were identified: *Staphylococcus aureus* ($n=7$), *Escherichia coli* ($n=3$), *Streptococcus pyogenes* ($n=3$), *Haemophilus* spp. ($n=2$), *Klebsiella oxytoca* ($n=2$), *Klebsiella pneumoniae* ($n=1$), *Moraxella catarrhalis* ($n=1$), and *Streptococcus milleri* ($n=1$).

Fourteen patients (32%) in the piperacillin/tazobactam group had positive blood cultures compared to 12 (27%) in the co-amoxiclav/aminoglycoside group. Three patients in the piperacillin/tazobactam group and one in the co-amoxiclav/aminoglycoside group harboured *Staphylococcus epidermidis*, which was considered a contaminant. In all four cases there was heavy growth of respiratory pathogens in the sputum cultures: *Streptococcus pneumoniae* ($n=2$), *Moraxella catarrhalis* ($n=1$), and *Haemophilus influenzae* ($n=1$). The 11 piperacillin/tazobactam patients with possibly true-positive blood cultures harbored the following microorganisms: *Streptococcus pneumoniae* ($n=8$), *Haemophilus influenzae* ($n=1$), *Escherichia coli* ($n=1$), and *Staphylococcus aureus* ($n=1$). The possibly true-positive blood cultures of the 11 aforementioned co-amoxiclav/aminoglycoside patients grew the following microorganisms: *Streptococcus pneumoniae* ($n=9$), *Streptococcus pyogenes* ($n=1$), and *Klebsiella pneumoniae* ($n=1$). Only one strain of *Staphylococcus aureus* in the piperacillin/tazobactam group was resistant to amoxicillin.

Table 3 Bacteriological efficacy in evaluable patients treated with either piperacillin/tazobactam or co-amoxiclav/aminoglycoside

	No. (%) of patients		P value
	PIP/TAZ group (n=25)	Co-AMX/amino group (n=27)	
Eradication (documented)	14 (56)	14 (52)	0.49
Eradication (presumed)	10 (40)	11 (40)	>0.5
Indeterminate	1 (4)	0	0.48
Persistence (documented)	0	1 (4)	>0.5
Persistence (presumed)	0	1 (4)	>0.5

PIP, piperacillin; TAZ, tazobactam; Co-AMX, co-amoxiclav; amino, aminoglycoside.

Not unexpectedly, seven strains of *Streptococcus pneumoniae* (1 in the piperacillin/tazobactam group and 6 in the co-amoxiclav/aminoglycoside group) were resistant to aminoglycosides.

The bacteriological efficacy at cessation of antibacterial therapy in the 52 evaluable patients is shown in Table 3. A favorable bacteriological response (documented or presumed eradication of the baseline pathogen) was observed in 24 of 25 (96%) evaluable patients in the piperacillin/tazobactam group and in 25 of 27 (92%) patients in the co-amoxiclav/aminoglycoside group ($P=0.53$). Persistence of the baseline pathogen at study cessation occurred in two patients in the co-amoxiclav/aminoglycoside group (both *Klebsiella oxytoca*, 1 case documented and 1 presumed).

Adverse Events. Four adverse events were registered: one in the piperacillin/tazobactam group and three in the co-amoxiclav/aminoglycoside group (2% vs. 7%; $P=0.32$). The only adverse event occurring in the piperacillin/tazobactam group – an elevated SGPT/SGOT serum level – was considered to be possibly due to the antibacterial regimen. In the co-amoxiclav/aminoglycoside group, acute renal failure ($n=2$) and fever of 38.5°C ($n=1$) were considered to be remotely or possibly drug related.

Discussion

The results of the present trial indicate that piperacillin/tazobactam is highly efficacious in the treatment of serious pneumonia in hospitalized patients. Its clinical efficacy was at least as good as co-amoxiclav plus aminoglycoside. Whereas the clinical cure rate was slightly (but not significantly) higher in the piperacillin/tazobactam group (81%) compared to the co-amoxiclav/aminoglycoside group (65%), the overall favorable response rate was equal with both regimens. Furthermore, the piperacillin/tazobactam regimen was as good as the comparator therapy with respect to the eradication of baseline pathogens.

Interestingly, although the severity of the pneumonia was comparable in both patient groups (Table 1), the mortality was lower in the piperacillin/tazobactam

(2.4%) than in the co-amoxiclav/aminoglycoside group (14%). The difference almost reached statistical significance ($P=0.059$). Thus, the mortality rate in the piperacillin/tazobactam group was quite low considering the fact that our study included only patients with serious pneumonia as defined in the Methods section. The possibly drug-related adverse events were non-significantly lower with the piperacillin/tazobactam treatment (2%) than with the co-amoxiclav/aminoglycoside regimen (7%; $P=0.32$).

The increasing prevalence of β -lactamase-producing microbes has reduced the clinical efficacy of piperacillin as a monosubstance [4]. The association of the β -lactamase inhibitor tazobactam with piperacillin restores the activity of the latter to a very considerable degree, as demonstrated by a number of trials [5-7] and by recent worldwide surveys indicating good *in vitro* activity of this combination against gram-negative and gram-positive aerobic and anaerobic organisms (A.P. Pallett and M.P. Carroll, 6th International Congress for Infectious Diseases, Prague, 1994, Abstract no. 857; M. Joshi et al. 6th ICID, Prague, 1994, Abstract no. 856) [10-13]. The spectrum of activity of piperacillin/tazobactam extends to most pathogens encountered in severe bacterial pneumonia. This combination has recently been investigated in two non-comparative trials in patients with mild to moderate pneumonia [8, 9]. In both of these studies, the combination therapy was found to be clinically and bacteriologically efficacious, well-tolerated and safe. The present trial provides evidence that this applies also to patients with serious pneumonia. Our results are in accordance with two other randomized, comparative trials that have shown that, in this same indication, piperacillin/tazobactam is as effective as cefuroxime (A.P. Pallett and M.P. Carroll, 6th ICID, Prague, 1994, Abstract no. 857), and more effective than ceftazidime (M. Joshi et al., 6th ICID, Prague, 1994, Abstract no. 856), regarding both clinical and microbial efficacy.

In these trials the safety assessments of the piperacillin/tazobactam association were also favorable, and the results were independent of the co-administration of aminoglycosides. These latter drugs are often administered in combination with other antibacterial substances for treatment of severe nosocomial infections. Their use is

associated with toxicity and increased costs for monitoring drug serum levels and renal function. Therefore, the development of equally effective combinations not containing aminoglycosides would be advantageous. The present study was designed to provide a direct comparison between piperacillin/tazobactam and a standard drug combination, namely co-amoxiclav plus an aminoglycoside. Our results indicate that the piperacillin/tazobactam combination compares favorably with co-amoxiclav plus an aminoglycoside. Moreover, costly monitoring of renal function and drug serum levels are not required with the piperacillin/tazobactam treatment.

In conclusion, the present clinical trial indicates that piperacillin/tazobactam is highly efficacious and safe for the treatment of serious pneumonia in hospitalized patients. It further demonstrates that this drug combination compares favorably with that of co-amoxiclav plus an aminoglycoside.

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